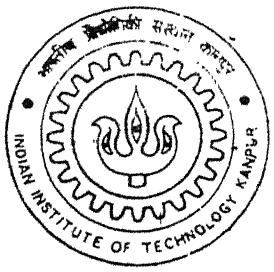
BIODEGRADATION OF ALKYLPHENOL ETHOXYLATES IN MINIATURISED BIOFILM REACTORS

By

Shweta Tripathi



Environmental Engineering and Management Programme

DEPARTMENT OF CIVIL ENGINEERING

Indian Institute of Technology Kanpur

MAY, 2002

BIODEGRADATION OF ALKYLPHENOL ETHOXYLATES IN MINIATURISED BIOFILM REACTORS

A thesis submitted in

Partial fulfillment of the requirement for the degree of

MASTER OF TECHNOLOGY

by

Shweta Tripathi

To the

Environmental Engineering and Management Programme
DEPARTMENT OF CIVIL ENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY, KANPUR
MAY, 2002

Thesis Work carried out at

ENGLER-BUNTE-INSTITUT, WATER CHEMISTRY DIVISION
UNIVERSITÄT KARLSRUHE (TH)

CERTIFICATE

This is to certify that the thesis entitled `*Biodegradation of Alkylphenol Ethoxylates in Miniaturised Biofilm Reactors*' by Shweta Tripathi is a record of work carried out by her under my supervision and that the work has not been submitted elsewhere for a degree.

Date: 13.05.2002

(Saumyen Guha)

Assistant Professor
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To

My Parents...

Acknowledgement

I express my heartfelt thanks to Dr. Saumyen Guha for his support and guidance during my entire thesis work. I am extremely grateful for his willingness to put things in the right perspective and to help me at all times irrespective of the time and place. This Thesis would not have been possible without his active help and support even when we were only in mail contact.

I am very thankful to Professor Dr Fritz H. Frimmel and Frau Gudrun Abbt-Braun for giving me this opportunity to work in their Laboratory at Engler-Bunte Institute, Karlsruhe. My sincere thanks to Margit Mueller for all her patience and support in guiding me and helping me in the experimental work. Without her patient answers to my endless queries it would have been almost impossible for me to understand the fine details of the work. Thanks are also due to Phillip Hoersch for all the help he rendered in biological analyses works.

I am thankful to DAAD for giving me this unique opportunity to do my thesis work under the guidance of two professors in different countries. The experience has been a singular one and has definitely added to my overall development.

All this would not have been possible without the blessings, support and constant encouragement from my parents. I am eternally thankful to them for making me what I am today.

Thanks are also due to Wolfgang, Daniella, Lenka and Christine for making my Lab a better place to work in. Without their friendly support it wouldn't have been possible to be familiar in an alien land.

Many thanks to Seema, Sonali, Shalini and Subhashis for their constant support and encouragement at every stage of my stay in IIT-Kanpur & abroad. Finally I express my thanks to all my other friends in IIT-Kanpur for making my stay here a memorable and eventful one.

Abstract

Alkylphenol ethoxylates (APEs) are one of the most widely used group of surfactants. The biodegradation of APEs has been a subject of interest from past two decades owing to the threats these surfactants are imposing on the natural ecosystem and livestock. The metabolites and degradation products of these surfactants have been found to be more recalcitrant and bioaccumulative than the parent compounds. Moreover, they also have been found to exhibit estrogenic character owing to their structural similarities with the estrogenic compounds.

The work involves study of biodegradation behavior of APEs of different ethoxylate chain lengths, present in a mixture of APEs. Analyses of degradation were performed with the help of High Performance Thin Layer Chromatography (HPTLC) coupled with Automated Multiple Development (AMD) technique. A comparative study of the total dissolved organic carbon degradation and degradation of individual APEs were conducted. Some of the intermediate products of degradation were isolated and identified. Efforts were made to identify and characterize, the biofilm microbiota into broad categories of bacteria using Fluorescent In situ Hybridization (FISH).

The APEs could be degraded both aerobically as well as anaerobically. The degradation was in the range of 80-85% in both aerobic as well as anaerobic reactors except for the short chain nonylphenol ethoxylates (NPEs). The short chain NPEs were degraded 100% in the anaerobic reactors. The major degradation products were identified as alkylphenol with 1 and 2 ethoxylate units. The FISH results indicate the presence of large populations of gram-negative bacteria (of phylum proteobacteria) in the both aerobic and anaerobic reactors. Small amount of archaebacteria were detected only in the anaerobic reactors.

The study results present an overview of the degradation pattern of different APEs and a comparative analysis intending to establish the relation between biodegradability of APEs and their chemical structure. The results indicate the significance of ethoxylate chain length in determining the biodegradability of the compounds. The study supports the

school of thoughts suggesting ethoxylate chain shortening as the major degradation pathway for the APEs existing in nature.

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List of Symbols

Symbol	Unit	Description
λ	[nm]	Wavelength
λ_{max}	[nm]	Absorption maxima
$\lambda_{excitation}$	[nm]	Excitation Wavelength
BAF	[-]	Bioaccumulation factor
BCF	[-]	Bioconcentration factor
BDOC	[-]	Biodegradable Dissolved Organic carbon
C_o	[mg/L]	Concentration of compound in organic phase
C_w	[mg/L]	Concentration of compound in water phase
DOC	[mg/L]	Dissolved Organic Carbon
EC_{50}	[mg.L]	Median Effective Concentration
i.d.	[mm]	Internal Diameter
K_{oc}	[-]	Organic Carbon partition coefficient
K_{ow}	[-]	Octanol Water Partition Coefficient
pK_a	[-]	Dissociation Constant of Acids
r.pm.	[-]	Rotations per minute
TOC	[mg/L]	Total Organic Carbon
$X_{constant}$	[mm]	Constant Distance between two development front in AMD

List of Abbreviations

Abbreviation	Description
AMD	Automated Multiple Development
AP	Alkylphenol
APE	Alkylphenol Ethoxylate
APEC	Alkylphenol Ethoxycarboxylate
APEn	Alkylphenol Ethoxylate n (where n = 1,2,3,4....)
APECn	Alkylphenol Ethoxycarboxylate n (where n = 1,2,3,4....)
BFR	Biofilm Reactor
DNA	Deoxy Ribonucleic Acid
ER	Estrogen Receptor
FISH	Fluorescent *In situ* Hybridization
GC	Gas Chromatography
HLB	Hydrophile Lipophile Balance
HPTLC	High Performance Thin Layer Chromatography
KHP	Potassium Hydrogen Phthalate
NP	Nonylphenol
NPE	Nonylphenol Ethoxylate
NPEC	Nonylphenol Ethoxycarboxylate
NPEn	Nonylphenol Ethoxylate n (where n = 1,2,3,4....)
NPECn	Nonylphenol Ethoxycarboxylate n (where n = 1,2,3,4....)
OP	Octylphenol
OPE	Octylphenol Ethoxylate
OPEC	Octylphenol Ethoxycarboxylate
OPEn	Octylphenol Ethoxylate n (where n = 1,2,3,4....)
OPECn	Octylphenol Ethoxycarboxylate n (where n = 1,2,3,4....)
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic acid
STP	Sewage treatment plant
TLC	Thin Layer Chromatography

Chapter 1

Introduction

1.1 Alkylphenol Ethoxylates

Alkylphenol Ethoxylates (APEs) are non-ionic surfactants made up of a branched chain alkylphenol that has been reacted with ethylene oxide to produce an ethoxylated chain. Commercial formulations are usually a complex mixture of homologues and isomers. The main alkylphenols used are nonylphenols (NP) and Octylphenols (OP). Nonylphenl Ethoxylates (NPEs) encompass about 80% of the world market, Octylphenol ethoxylates (OPEs) comprise 15% and the others represent the remaining 5%(Renner, 1997).

APEs have been in use for more than 40 years and account for nearly 6% of the worldwide use of surfactants (Nimrod, 1996). They are used as detergents, emulsifiers, and wetting agents, dispersing agents in household products and in agricultural and industrial applications. In United States, industrial uses of APEs encompass the largest category (55%); institutional cleaners comprise 30% of the total and household cleaning and personal care products make up the rest (Renner, 1997).

Concerns about these surfactants arose in the early 1980s in Europe when a group of Swiss researchers discovered a twist in their environmental fate. The surfactants were inadvertently being transformed into more toxic compounds during the biodegradation that accompanies wastewater treatment. Particularly high levels of Alkylphenols (APs) were measured in digested sewage sludge. The metabolites were also shown to be more

persistent and more lipophilic than the parent APEs, a finding that raised concerns about bioaccumulation of the toxic compounds.

Removal of APEs depends to a large extent on their degradation and transformation by the bacterial population in the environmental systems. The bacterial transformation of the compound leads to production of new compounds much different in characteristics from the parent compound. This puts a question mark on the environmental acceptability on the transformation metabolites.

APEs can be biologically degraded in sewage treatment plants and in the natural environment. However, some of the degradation products, including alkylphenols, are more persistent and more lipophilic than the parent surfactants, and degradation products are found in receiving waters of sewage treatment plants. It has been estimated [Ahel, 1994], that of all the nonylphenolic compounds that enter the STPs, about 60 to 65% are released to the environment (as metabolites or parent compounds) via the secondary effluents (40%) and the sewage sludge (20%). The biotransformation and biodegradation metabolites comprising of lower ethoxylates, carboxylates and the alkylphenols are much more toxic and bio-assimilable as compared to the parent compounds [Servos, 1999]. Moreover, many of the metabolites were also reported to exhibit estrogenic character.

In the former decade the studies on biodegradability of APEs were centered on assessment of its ultimate biodegradation. However, due to arising concerns, need arises to have a knowledge about the spectrum of resultant transformation metabolites and also the intermediate degradation products being formed, so that efforts can be implemented to channel the degradation pathway for the production of most environmentally acceptable transformation products.

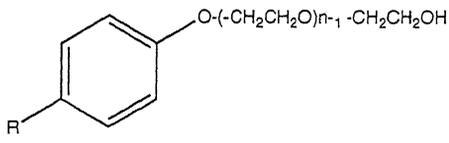
Chapter 2

Literature Review

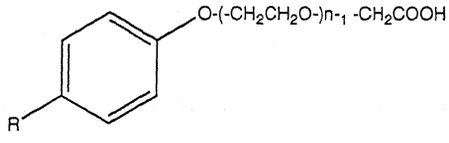
2.1. Chemical Structure of APEs

APEs are amphiphilic compounds. They can be structurally divided into two basic moieties – a hydrophobic moiety and a hydrophilic one. The hydrophobic part comprises of an alkylphenol, which is formed by alkylating a phenol. The alkyl chain attached to the phenol can be a straight chain as well as a branched alkyl chain depending on the synthesis reaction conditions and the parent compound used for alkylating the phenol. The predominant positional isomer of alkylphenols (used for APE synthesis) is the para-isomer, which usually comprises $\geq$ 90% of industrial formulations, while the ortho-isomer comprises $\leq$10% (Maguire, 1999). The hydrocarbon alkyl chain (branched or unbranched) most commonly contains eight (Octylphenol Ethoxylates, OPE) or nine (Nonylphenol Ethoxylates, NPE) carbon atoms. The hydrophilic moiety of APEs however, comprises of a polyethoxylate chain (E-chain) with varying ethoxylation degrees (number of ethoxylation (E) units, n = 1 to 100).

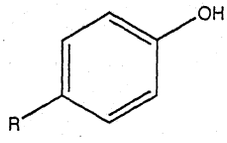
APEs undergo biotransformation in both aerobic and anaerobic environments to yield a variety of transformed products, which comprise of lower ethoxylates, alkylphenol carboxylic acids and the non-ethoxylated alkylphenols. Besides these products, halogenated (on the ring) derivatives of the alkylphenol ethoxylates and carboxylates have also been found in the effluents of some sewage treatment plants that employ Chlorine for disinfection. The structure of APEs and some of their degradation products are shown in the figure 2.1.



Alkylphenol Ethoxylates (APE n)



Alkylphenol Ethoxycarboxylate (APEC n)



Alkylphenol (AP)

R = C_8H_{17} (Octyl), R = C_9H_{19} (Nonyl), R is usually branched

Figure 2.1. The structures of Alkylphenol Ethoxylates and related compounds.

2.2. Properties and Implications with respect to Environmental Pathways

The APEs have been widely used in the last 40 years in large quantities. The high consumption and a unique transformation pathway of alkylphenol ethoxylates (Giger et al, 1984; Ahel, 1987) contributes to the potentially high environmental hazard associated with these compounds. Several investigations have shown that during alkylphenol ethoxylate biotransformation, various highly persistent metabolites are formed. It has been estimated that about 60% of nonylphenol ethoxylates entering biological sewage and sewage sludge treatments are subsequently released into the environment, 85% being in the form of metabolic products (Ahel, 1987)

Some of the transformation products of NPEs have a pronounced lipophilic character, a high bio-concentration factor (BCF) and their toxicity to aquatic life is much higher than the toxicity of the parent compounds. These compounds have also been reported to act as endocrine disruptors, which increases the environmental threat and concern.

In light of so many hazardous potentials of the APEs and their metabolites, it becomes essential to have a light on some of their physical and chemical properties, which have bearing on the environmental persistence of APEs.

2.2.1. Lipophilicity

Several attempts have been made to correlate lipophilicity of APEs with toxicity and bioaccumulation data and it has been observed that the more lipophilic oligomers are more toxic chiefly because they are more bio accumulative in the body of organisms. A convenient way to express the lipophilicity of a compound is to determine the *octanol-water partition coefficient*, K_{ow}, defined as follows:

$$\textit{Octanol-water Partition co-efficient} = \frac{\textit{Concentration of the test compound in the organic phase}}{\textit{Concentration of the test compound in water phase}} \quad \text{Eq.2.1}$$

The Octanol-water partition co-efficients of some APEs can be seen in table 2.1

Compound	Log K_{ow}
NP	4.48
NPE-1	4.17
NPE-2	4.21
OP	4.12

Table 2.1. Octanol-Water partition coefficient for some APEs

In the absence of experimental data, the partition coefficients for higher oligomers are often calculated on the basis of correlation with aqueous solubility. These are shown in table 2.2.

No. Of E-Groups per molecule	**Log K_{ow}**	
	OPE	**NPE**
0	3.9	4.2
1	4.1	4.4
2	4.0	4.1
10	3.6	4.1

Table 2.2. Log K_{ow} values based on aqueous solubility (adapted from Ahel et.al,1993b).

The relatively high values obtained for higher oligomers indicate that in spite of a significant share of the hydrophilic groups; the basic lipophilic character of these compounds is preserved.

2.2.2. Aqueous Solubility

The APE solubility in water is based on the hydration of ether functional groups through hydrogen bonds *i.e.* more the degree of ethoxylation, more is the solubility of APE in aqueous medium. Table 2.3 gives the water solubilities of a few APEs.

Compound	Solubility (mg/L)	Compound	Solubility (mg/L)
NP	5.43	OP	12.6
NPE-1	3.02	OPE-1	8.0
NPE-2	3.38	OPE-2	13.2
NPE-3	5.88	OPE-3	18.4

Table 2.3. Solubilities of APs and APEs in water at 20.5°C (Taken from Ahel, 1993a).

Also, in general, the solubility of the non ionic surfactants decreases with increasing *temperature (hydration reduces with temperature).* For APEs the effect is less pronounced for lower oligomers since their molecules bind considerably less water.

2.2.3. Colloidal and Interfacial Chemical Properties

The physical and chemical properties of APEs like all other surfactants in general, are influenced by their amphiphilic molecular structures. At very low concentrations APE adsorb at interfaces and reduce the surface tension, however, with increasing concentrations, the formation of micelles in the bulk phase starts to occur. The CMC values for APEs are in the range of 10^{-3} to 10^{-5} $mol.L^{-1}$ (Thiele et al,1997). The CMC value for the commercial surfactant Triton X-100 (OPE 9.5) is about 43 mg/L (Guha and Jaffe,1996). This property of APEs makes them efficient to be widely used surfactants worldwide.

2.2.4. Adsorption on soil

APEs and specially their degradation products are liable to reach the soils through the application of sewage sludge onto the soils. The behaviour thereafter depends on the affinity of the compound for the soil. This can be assessed through the *organic carbon partition*

coefficient, K_{oc}. It is a measure of tendency for organic substances to be adsorbed by soil and sediments and is expressed as :

$$\textit{Organic Carbon Partition coefficient} = \frac{\textit{Amount of chemical adsorbed per kg of organic carbon}}{\textit{Amount of chemical dissolved in one litre of solution}} \quad \text{Eq. 2.2}$$

The values for OP and NP are given in table 2.4:

Compound	K_{oc}
NP	32,400
OP	18,000

(Taken from Toxnet,2000)

Table 2.4. K_{oc} values for APs

Based on such a high K_{oc} values, it can be safely said that if released to soil, NP and OP will have almost no mobility and if released to water, AP should strongly adsorb to suspended solids and sediments. (Toxnet, 2000).

2.2.5. Bioaccumulation

The transformation products of NPEs such as NP, NPE 1 and NPE 2 have a pronounced lipophilic character and therefore, bioaccumulate in aquatic organisms chiefly in the adipose tissues

There are two ways of studying /expressing the bioaccumulation potential of compounds; *bioconcentration factor* (BCF) or *bioaccumulation factor* (BAF). At a steady state,

$$BCF = \frac{\textit{Concentration of chemical in the tissues of an organism}}{\textit{Concentration of the chemical in water}} \quad \text{Eq 2.3}$$

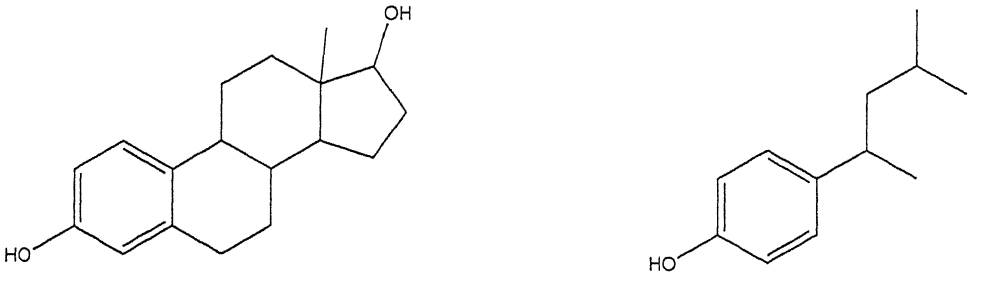
In general, the BCFs of organic compounds increase with increasing K_{ow} and with decreasing solubility in water, *i.e.* less is the degree of ethoxylation, more is the BAF of the surfactant. Also based on structural similarities and lower log K_{ow} values, OP and OPEs are predicted to have BCFs and BAFs slightly lower than NP and NPEs (Servos, 1999).

2.2.6. Toxicity

The toxicity of AP increases as the length of hydrophobic chain increases (Mc Leese etal, 1981). Toxicity may occur by partition into lipid membranes in the organisms, for example mitochondrial membrane, leading to the uncoupling of energy production (Argeese et al, 1994). The toxic effects of APEs normally start at concentrations more than 1.5 mg/L (Warhurst, 1995).

2.2.7. Estrogenic Potential

Environmental estrogens or xenoestrogens are exogenous estrogenic compounds that mimic the estrogenic action of estrogens in organisms. APEs and AP are believed to be xenoestrogens that compete with estradiol for Estrogen Receptors (ER). This property is attributed to the chemical structure of APEs and AP. It is now known that many environmental estrogens possess a para substituted phenolic group (Figure 2.2).



17 β- estradiol Alkylphenol

Figure 2.2. Structures of the natural estrogen 17 β estradiol and the alkylphenols.

The presence of more than one phenolic group can render a compound more estrogenic. While the phenolic group fits into the binding pocket of the estrogen receptor closely, the rest of the molecule plays an important role in determining whether the compound will act as an a agonist or antagonist. Since estradiol is the most common endogenous estrogen, the estrogenic activity of a compound is usually expressed relative to the activity of estradiol (table 2.5)

Compound	Relative Potancy[a]	ER binding affinity[b]
Estradiol	1.0	1.0
4-tert-Octylphenol	0.00037	0.0007
Nonylphenol	0.0000090	0.0003
NPE-2	0.0000060	ND
NPE-9	0.0000002	-
NPEC-1	0.0000063	0.00005

a, Data from rainbow trout in vitro hepatocyte bioassay, Nimrod and Benson,1996
b, Data from assays on rainbow Trout liver, Nimrod and Benson,1996
ND, indicates receptor binding was not detected
-, no data given

Table 2.5. Potencies of some Xenoestrogens relative to estradiol

2.3. Biodegradation Of APEs

The degradation of a chemical compound due to metabolic activities of living organisms is reckoned as Biodegradation. The process of biodegradation solely depends on the susceptibility of the compound in question to the living organisms in its surrounding. This behaviour of any chemical compound is influenced by many factors including its physical and chemical properties. However, the biodegradation of any compound and its persistence capability are also affected by the ecosystem specific properties such as the nature and concentration of microbial population, the nature and concentration of dissolved and suspended materials, temperature of the system and degree of insulation etc. In general, important physical, chemical and biological removal mechanisms for any chemical in any ecosystem are:

i. Volatilisation

ii. Adsorption onto suspended solids and sediments.

iii. Chemical or photochemical degradation or transformation, and

iv. Uptake and transformation by micro organisms i.e., biodegradation

The importance of such pathways for specific chemicals depends on the chemical and the ecosystem. In our system the chemical compound of interest are surfactants, specifically APEs and their metabolites and the living organisms of interest are microbes present in the environment receiving the wastewater, specifically bacteria. One of the important characteristics of bacteria is its adaptability to the changing surroundings. Owing to this metabolic versatility of bacterial groups, most organic compounds are degradable by the bacteria after a shorter or longer period of exposure. The major three phenomena responsible for this bacterial acclimation to the surroundings are:

- Population Shift
- Appearance of new genotypes
- Enzyme induction

However, whatever be the way of bacterial adaptation to the surroundings, the overall process of biodegradation is always oxidation of the substrate. Bacteria utilise the energy so released for building up new protoplasm or as fuel for life processes and in turn the compound is broken down to its smaller and structurally different metabolites.

2.3.1. Types of Biodegradation

Biodegradation has been defined in many different contexts, the definition depending primarily upon the parameter being utilised for measuring biodegradability. Swisher (1987) has indicated definitions for two degrees of biodegradation depending on the resultant end products :

2.3.1.1. Primary Degradation

This type of degradation is said to have occurred when the parent molecule has been oxidised or otherwise altered by bacterial action to such a extent that those

characteristic properties are no longer evident or when it no longer responds to analytical procedures more or less specific for detecting the original surfactant. For the APEs this step is relatively rapid and results in the degradation intermediates AP, APE1, APE2 etc

2.3.1.2. Ultimate Biodegradation

This type of biodegradation is defined as the complete conversion of the surfactant molecule to carbon dioxide, water, inorganic salts and the products associated with the normal metabolic processes of the bacteria. The conversion of organic matter to CO_2 and H_2O is also referred to as mineralisation and ultimate biodegradation is often considered equivalent to complete mineralisation. The ultimate biodegradation of APs and lower ethoxylates occurs more slowly because of the presence of benzene ring and the compounds' water insolubility.

2.3.2. Effect of Chemical structures on Biodegradability

Biodegradability of Alkylphenol Ethoxylates depends on two major structural factors:

1. The length of ethoxylate chain
2. The structure of hydrophobic group.

It is generally seen that the biodegradability is promoted by the increased hydrophobe linearity and deterred by hydrophobe branching, particularly by terminal quaternary branching. Also, it is seen that biodegradation decreases with the increased length of Ethoxylate chain. This has been attributed to the reduced lipophilic properties and increased molecular size of the higher ethoxylates leading to the poorer transportation across the cell membrane [Swisher, 1987] Also ortho isomers are more resistant than para and hence they

show poorer degradability and greater resistance to shortening of the ethoxylate chains (Maguire, 1999).

2.3.3. Fate of APEs in the Environment

APEs, NPEs and OPEs in particular, are High Production Volume (HPV) chemicals i.e., they have annual production or importation volumes above 1 million pounds. As such they are released to the environment in very large quantities. The chemicals are first released to the sewage treatment plants (STPs) where they undergo transformation and finally reach water bodies and soil through the discharge of effluents and sludge application onto the soil, respectively.

2.3.3.1. Degradation and Persistence in Sewage Treatment Plants (STPs)

The APEs are released into wastewaters where they undergo transformations and are subsequently released to the river bodies and the soil. The concentrations of NPE oligomers and NP in wastewater influents have been seen to vary from 37 - 123 μg/L to 5700 – 9000 μg/L [Thiele, 1997]. Thorough investigations have been carried out on the fate of the NPEs in the different steps of sewage treatment [Ahel, 1994a]. Figure 2.3 summarizes the biodegradation/biotransformation scheme for the APEs most often encountered in the sewage treatment plants

During activated sludge treatment, the higher oligomers (n>8) totally disappear in favour of their metabolic products NP, NPE1 and NPE2 which are resistant to further microbial transformation and are partially discharged to the aquatic environment via secondary effluents. Most of the NP, NPE1 and NPE2 are, however, removed from the treated wastewater by adsorption on the sludge because they are more lipophilic than the parent compound. During anaerobic sludge stabilization, NPE1 and NPE2 are further biodegraded to NP, resulting in very high NP levels (450 – 2350 mg/kg) in digested sludge [Thiele,1997].

The sludge is most often disposed off on land. According to Ahel (1994a) starting from 100% NPEs (70% NPEs, 20% NPE-1/ NPE-2) in the raw sewage 60% of these compounds (19%as NPECs,11% as NPE-1 or 2, 25% as NP and 8% as untransformed NPEs) are released into the environment via secondary effluents (40%) and digested sludge (20%).

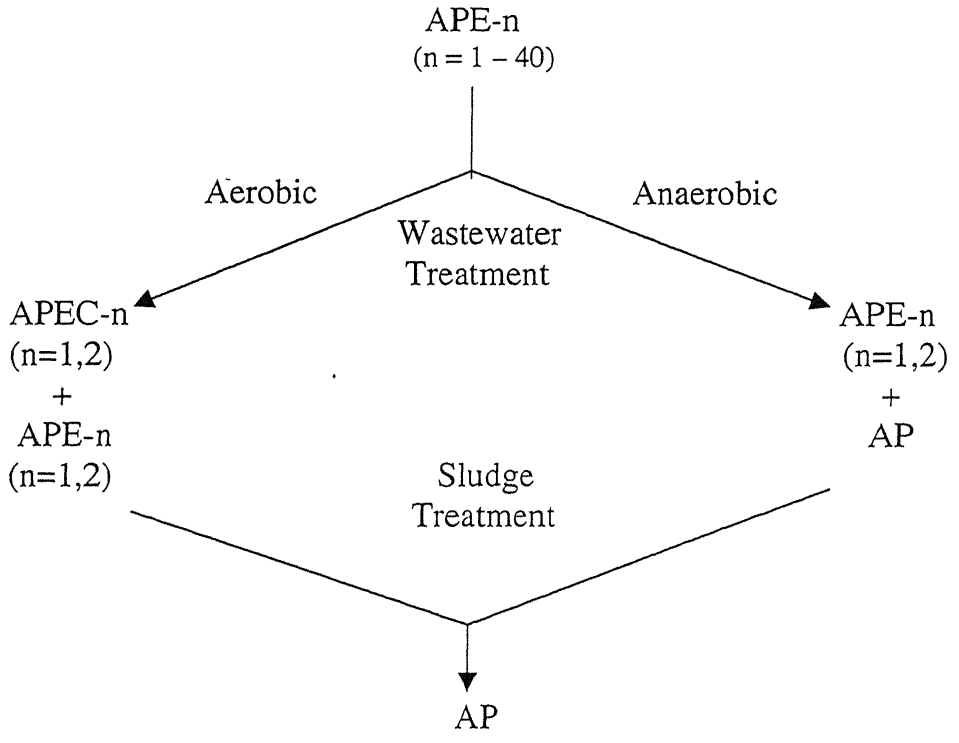


Figure 2.3. Aerobic and Anaerobic Biotransformation pathways of APEs

(Adapted from Thiele, 1997)

2.3.3.2. Degradation and Persistence in Aquatic Ecosystems

There are relatively few studies of the persistence and fate of NPEs and NP in aquatic ecosystems compared to the number of studies on biodegradation in STPs. In Aquatic Ecosystems, it appears that parent NPEs are not persistent, but some degradation products may have moderate persistence, especially under anaerobic conditions. The U.K.

Environment Agency [1997] estimated a half-life for biodegradation in surface water of about 30 days.

Microbial acclimation to such chemicals is required for optimal degradation efficiencies. Sunlight photodegradation of such products is also expected to be important in Aquatic Ecosystems. The primary degradation of NPEs is faster than ultimate degradation of more persistent products such as Nonylphenol 1 Ethoxylate, Nonylphenol 2 Ethoxylate, Nonylphenol 1 Ethoxycarboxylate, Nonylphenol 2 Ethoxycarboxylate) and Nonylphenol (NP). The recent studies indicate that NP can be moderately persistent in the sediments [Heinis et al., 1998]. There is a need for mass balance studies that take into account the relative importance of biodegradation and sunlight photodegradation, as well as adsorption to suspend solids and bed sediment, aerobic and anaerobic conditions, and temperature effects. To the extent that NPEs are used in aerially applied pesticide formulations, there is a need to determine their atmospheric chemistry, photochemistry and fate.

2.3.3.3. Degradation and Persistence in Groundwater and Soil

There are relatively few studies done on the persistence of APEs and AP in porous media compared to the aquatic ecosystems. However, based on the limited data available, NP and lower ethoxylates and carboxylates are persistent in groundwater. They are also persistent in landfills under anaerobic conditions, but they do not appear to be persistent in soil under aerobic conditions. The U.K. Environment Agency [1997] estimated a half-life for biodegradation in soil of about 30 days.

2.4. The Biofilm Reactors

A biofilm is a layer-like aggregation of microorganisms attached to a solid surface. In many aquatic systems, especially those having a high specific surface area and low nutrient concentrations, biofilm constitute 90 to 99.9 % of the bacteria. Biofilms are found in or on

streambeds, groundwater aquifers, lake benthos etc. Engineered processes that utilize films of bacteria include trickling filters, rotating biological contractors etc.

Unexpected changes in the quality of wastewaters usually result in difficulties concerning the biological wastewater treatment processes. The characterizing of an existing pollution requires fast and reliable analytical techniques to allow proper engineering operations. Small biofilm reactors (BFRs) in combination with determination of dissolved organic carbon (DOC) have been shown to be effective tools for rapid characterization of the biological elimination of organic compounds in water [Hesse, 1999].

The biofilm reactors have been used by several researchers [Kaplan, 1995; Wild, 1999] to study the biodegradation of environmentally relevant chemicals. BFRs have been used as model systems to assess the degradability of pharmaceuticals (ibuprofen, diclofenac etc) during sewage treatment (Zwiener, 2000). In a comparative study of the performance of the BFRs and a pilot sewage treatment plant, the aerobic BFRs were found to function as well as the pilot sewage plant. Thus, a good co-relation between the performance of a conventional wastewater treatment set up and that of the BFRs has been found to exist (Zwiener, 2000). Hence, BFRs are a faster way of assessing the behavior of chemicals in actual wastewater treatment plants. Alternatively once the reactors have been acclimatized to a particular compound and a steady state has been reached, they can also be used as a check on the quality of the influent. Any alteration in the quality of the influent will produce an adverse effect on the performance of the bacteria, which in turn can be seen in the altered characteristics of the effluent. Within a few hours it is possible to evaluate the current situation of elimination process and to control the wastewater treatment efficiency.

2.5. Scope of present work

Removal of APEs depends to a large extent on their degradation and transformation by the bacterial population in the environmental systems. The bacterial transformation of the compound leads to production of new compounds much different in characteristics from the parent compound. This puts a question mark on the environmental acceptability on the transformation metabolites.

APEs can be biologically degraded in sewage treatment plants and in the natural environment. However, some of the degradation products, including alkylphenols, are more persistent and more lipophilic than the parent surfactants, and degradation products are found in receiving waters of sewage treatment plants. It has been estimated [Ahel, 1994], that of all the nonylphenolic compounds that enter the STPs, about 60 to 65% are released to the environment (as metabolites or parent compounds) via the secondary effluents (40%) and the sewage sludge (20%). The biotransformation and biodegradation metabolites comprising of lower ethoxylates, carboxylates and the alkylphenols are much more toxic and bio-assimilable as compared to the parent compounds [Servos, 1999]. Moreover, many of the metabolites were also reported to exhibit estrogenic character.

In the former decade the studies on biodegradability of APEs were centered on assessment of its ultimate biodegradation. However, due to arising concerns, need arises to have a knowledge about the spectrum of resultant transformation metabolites and also the intermediate degradation products being formed, so that efforts can be implemented to channel the degradation pathway for the production of most environmentally acceptable transformation products.

Chapter 3

Objective of Present Work

The present work aims to study the biodegradation mechanism of APEs, and to study the composition of the microorganisms in the bio-films. The work comprised of the following stages:

1. An assessment of degradation pattern of APEs with different chain lengths was pursued with the help of miniaturized bio-film reactors (hereafter, referred to as BFRs)
2. Investigations on the composition of bacterial consortium comprising the biofilms in the BFRs were done using Fluorescent *In situ* Hybridization (FISH).
3. Efforts were made to analyze the APEs and their metabolites removal by adsorption on the surface of biofilms in the reactors.

The studies were done for two Nonylphenol ethoxylate mixtures (NPE), long and short ethoxylate chains and for Octylphenol Ethoxylates (OPE), both under aerobic and anaerobic condition. The selected APE compounds were Marlophen NP10 (mixture of NPEs with average n = 10), Marlophen NP3 (mixture of NPEs with average n = 3) and Triton X-100 (mixture of OPEs with average n = 9.5).

The following parameters were monitored throughout the experiment:

- Concentration of APE in parent compound (influent of BFRs).
- Concentration of APEs after degradation (effluents from BFRs).
- Formation and concentration of degradation products.
- DOC concentration before and after analyte degradation.

In addition, the following parameters were determined at the end of experiment –

- Biomass contents in the BFRs.

- Extent of removal of analytes and their metabolites by adsorption onto the biofilms.

Based on the data from BFR studies, an assessment of the possible pathway and mechanism for APE biodegradation in both oxic and anoxic conditions in the BFRs was interpreted.

Chapter 4

Materials and Methods

4.1. Overview of the Study Design

It was endeavored to study the biodegradation pattern of three different compound mixtures of Alkylphenol Ethoxylates viz. Marlophen NP10 (Long chain NPEs), Marlophen NP3 (Short chain NPEs) and Triton X-100 (long chain OPEs) under both aerobic and anaerobic conditions with the miniaturized biofilm reactors. Biodegradation was measured as the decrease in the quantity of individual ethoxylates in the ethoxylate mixtures, by comparing the influents and the effluents from the biofilm reactors and the Dissolved Organic Carbon (DOC) in the reactor effluents as compared to that in the influents. The individual ethoxylates in the sample were determined by the High-Performance Thin Layer Chromatography (HPTLC). Since the APEs are found in high concentrations in wastewaters, it was decided to perform the experiments in a wastewater matrix. Also efforts were made to identify the bacterial population in the biofilm inside the reactors. For this, the Fluorescent In situ Hybridization (FISH) technique was adopted and the bacterial species present in the consortium in biofilms were analyzed for their specific groups using the appropriate probes.

4.2. Analytical Methods

The entire experimental analysis was performed using three major analytical methods, *viz.* High Performance Thin layer Chromatography coupled with Automated Multiple

Development (HPTLC/AMD) for separation and detection of the individual ethoxylates from their compound mixtures; Dissolved Organic Carbon (DOC) analysis to monitor the biodegradation in terms of DOC reduction in the feed to the reactors and Florescent *In situ* Hybridization (FISH) technique for studying the microbial ecology in the Biofilm reactors

4.2.1. High Performance Thin Layer Chromatography (HPTLC)

The TLC, or high performance TLC (HPTLC), is primarily used as an inexpensive method for separation, for qualitative identification, or for the semi-quantitative visual analysis of samples. Recent reviews show that the TLC and HPTLC techniques can be used to solve many qualitative and quantitative analytical problems in a wide range of fields, including medicine, pharmaceuticals, chemistry, biochemistry, food analysis, toxicology and environmental analysis. The basic principle involved in the TLC technique is partition of compounds from their mixture on the basis of their affinity for a mobile phase solvent and for the thin stationary phase layer. Stepwise and multiple development are among the methods most commonly used. Their advantages can be increased by the use of properly designed and applied instrumentation. This is true in AMD, which has the added benefit of gradient development. The whole AMD process is completely automated. It begins with a drying step, involving evacuation of the specially designed chromatography chamber, in order to remove from the HPTLC plate, any traces of solvent from the standard and sample solutions, which have been applied. There can be interference with the chromatography if this drying process is omitted.

The chamber is then ventilated with a clean, inert atmosphere of controlled composition (N_2 gas) before the first run is commenced by the introduction of mobile phase into the tank. This run is terminated after a few seconds as the mobile phase is sucked from the tank with a pump. The next cycle now commences with drying ventilation and a somewhat longer period of development, again followed by the removal of the mobile phase. This process is continued, until the entire development programme has been worked through. The final step is always drying of the chromatogram so that the HPTLC plate is never removed from the chamber in a moist state. This means that the workstation is not subject to contamination by solvent vapors, an advantage - which should not be underestimated for routine operation, particularly in the field of environmental analysis. The HPTLC/AMD system offers several advantages of which the particular one is the stepwise separation of different chemical and also the focusing of the developing band after every phase of instrumental run. This helps in sharp separation of more than two compounds of a mixture and also ensures high reproducibility of the results.

4.2.2. Dissolved Organic Carbon Analysis

DOC is measured as NPOC (non purgeable organic carbon, NPOC). The samples are acidified and sparged automatically with high purity air to eliminate inorganic carbon (IC) prior to measurement of total carbon (TC) concentration. The IC is converted to CO_2 and is removed. The measurement thus obtained in NPOC indicates non-volatile carbon, which does not get eliminated in the sparging process. The detection of TC is via

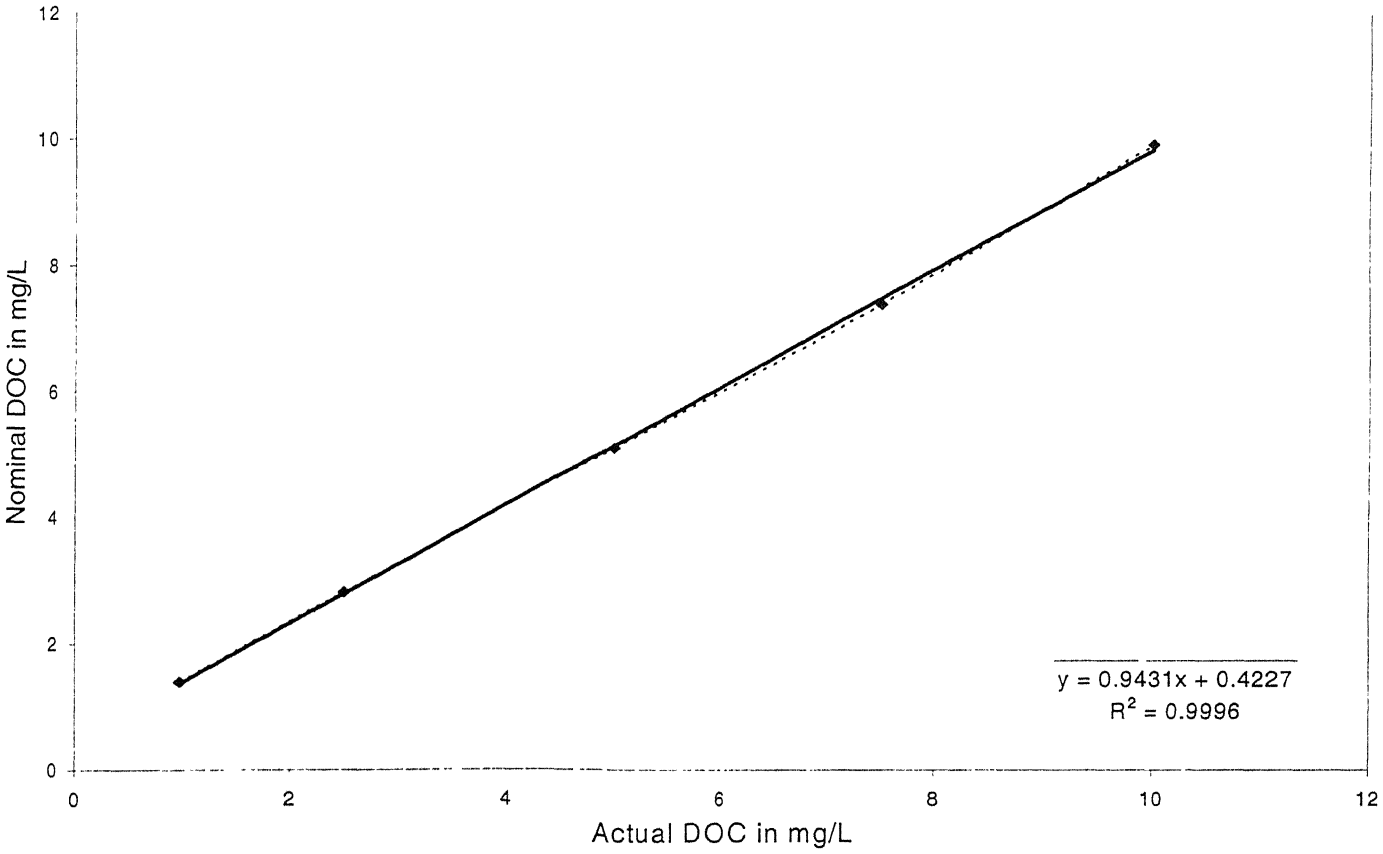


Figure 3.1. Calibration curve for TOC using external KHP standards on Shimadzu TOC analyzer.

high temperature combustion. The sample is introduced to the combustion tube (which is heated to 680 °C and is filled with oxidation catalyst) where the TC component is decomposed or combusted to CO_2. This is then sent to the non-dispersive infrared gas analyzer (NDIR) where CO_2 is detected. The NDIR output is a detection signal (analog signal), which generates a peak whose area is calculated by a data processor.

The method of external calibration was followed for the measurement of sample TOC. Potassium hydrogen phthalate (KHP) was used as the standard compound. A series of five standards was made with concentrations of 1.0, 2.5, 5.0, 7.5, and 10.0 mg/L from a stock solution of 1000 mg/L (as TOC). Both the stock solution and the dilutions were made in Milli Q. The series of standards was measured with every test run. Both samples and standards were measured in duplicate sets in every run. Very good correlations between the actual and measured standards were normally achieved (Figure 3.1). In general, very little day-to-day variation between the correlation parameters was observed.

4.2.3. Fluorescent In situ Hybridization (FISH).

The advent of fluorescent dyes for use as labels has made it possible to visualize multiple probes at the same time. Fluorescence in situ hybridisation, or FISH, is a method used to label cells or chromosomes according to the sequences of nucleic acids contained within them. The advantage that this method offers is the capability to visualise more than one type of probe hybridised to the same cell, hence revealing different properties of the cell simultaneously. In microbiology, the nucleic acid that is labelled is usually the RNA of the ribosomes and the target is usually the whole cell. The process works by taking

fluorescent-labelled pieces of DNA or RNA called probes that are around 20 nucleotides in length. The probes are incubated in the presence of cells under appropriate conditions to permit specific hybridisation of probe to target nucleic acid. Cell types that contain ribosomes with complementary RNA sequences become labelled by the binding of the fluorescent probe *in-situ*. These labelled cells can then be visualised by flow cytometry or fluorescence. The advantages of the FISH technique are multifarious. A practical problem in the identification of microorganisms in activated sludge and biofilms is that the majority of the biochemical tests can only be performed on pure cultures. This requires isolation and culture of the individual strains, which is often not possible, since the growth requirements of the microorganisms involved are not always known. In contrast with cultivation-dependent methods, *in-situ* hybridisation techniques have the potential to obtain a more complete view of the diversity and dynamics of the microbial consortia involved in the activated sludge process. With rRNA probes the number of detectable cells is higher than with any other technique. The rRNA approach is unbiased by the limits of pure-culture techniques. Cultivation dependent methods detect only up to 10% of the real existing bacterial population in an environmental sample. The FISH technique allows the specific detection up to 90% of the whole bacterial population.

4.3. Materials

For performing the entire biodegradation analysis, a large number of chemicals and instruments were used. The chemicals, their source and purity are listed in Table 7.1 of Appendix I. The instruments used and their specification are listed in Table 7.2 of Appendix I. Synthetic Wastewater (SWW) was prepared according to DIN 38412, OECD-Guideline 303A/91 and the composition is shown in Figure 3.2

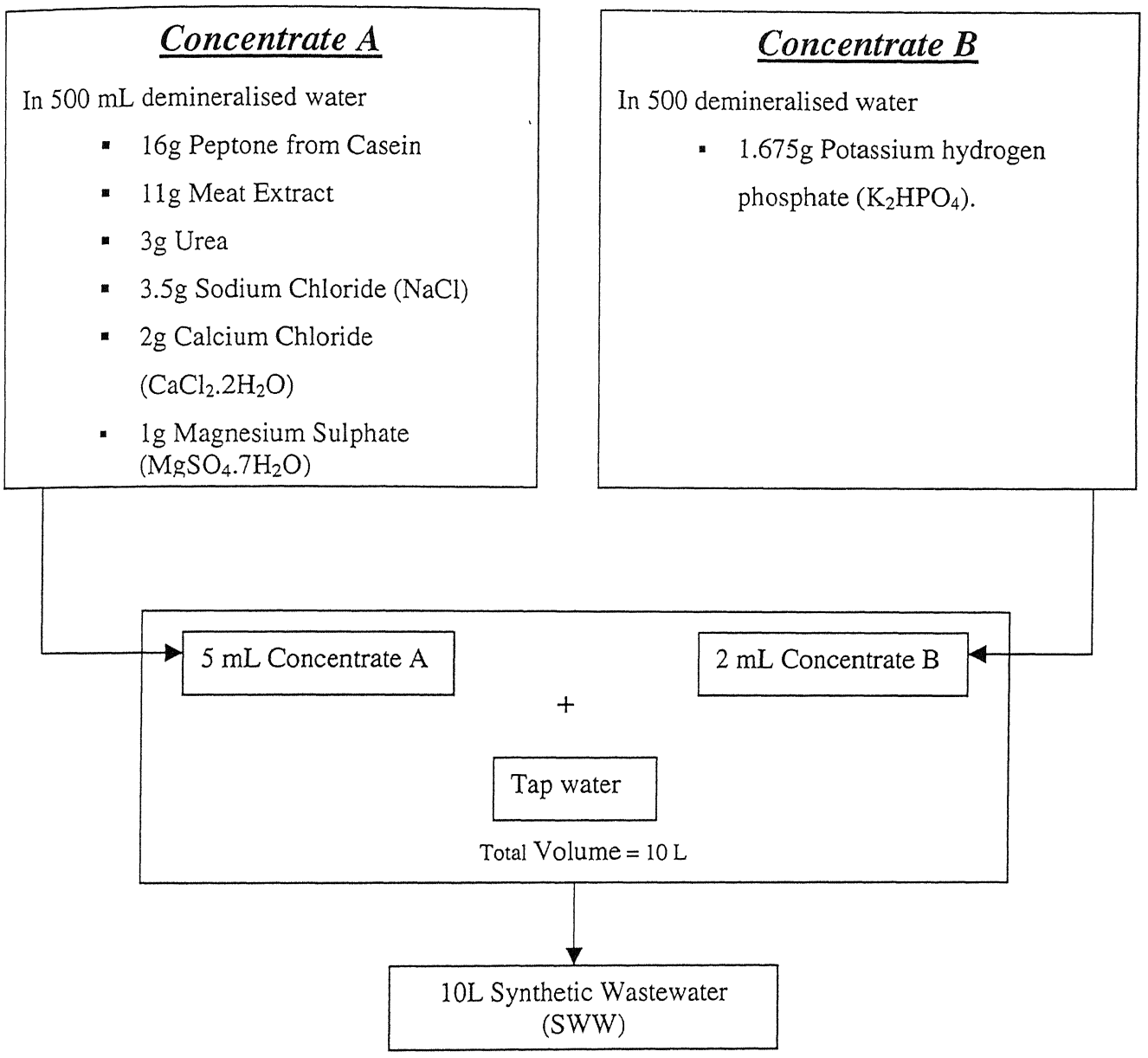


Figure 3.2. Method for preparing Synthetic wastewater

4.4. Experimental Methods

The entire experimental phase was divided into three different phases *viz.* the pre-tests, development of biofilm reactors and the biodegradation of APEs. The pre-tests were conducted so as to ensure that the BFRS are being properly cultured and that accuracy in calculations of results and data interpretation is achieved.

4.4.1. Pre Tests

Prior to starting the experiment with the biofilm reactors, some experiments were conducted to decide the exact procedure to be employed in the tests. The details of these pretests are described in the following sections.

4.4.1.1. Adsorption of the analyte onto the container walls

In order to minimize the error in results owing to adsorption of the test compounds on the walls of the sample container, tests were conducted to know the sample adsorption on the container walls (if any) with respect to increasing time. This was done by monitoring any appreciable decrease of the concentration of test compounds in sample with time.

Two 5L glass bottle were filled with 2L of solution each. The solution contained 5-mg/L solution of Marlophen NP3 in Synthetic Wastewater (SWW) matrix. The bottles were kept undisturbed in same conditions as for feeding analytes later on during the test phase and sampling was done from the center of the bottle with the help of glass pipette at time intervals shown in table 4.1

Sample No.	Time from starting time.
1	30 min
2	1h, 30min
3	3h, 30min
4	8h, 30min
5	24h

Table 4.1. Sampling time from the beginning of the adsorption experiment.

The samples were extracted using Solid Phase Extraction (SPE) immediately after sampling. The extracts were then analyzed using High-Performance Thin Layer Chromatography (HPTLC) to determine the actual concentration of analyte in the samples.

The concentration of all the NPEs under consideration for quantitative analysis were not found to change by appreciable amount with time. Figure 3.3 shows the relative concentrations of individual ethoxylates with respect to original sample at different intervals of time.

The concentrations at one interval of time were found to be low but the later concentration values were again high. Moreover, a high standard deviation value was found to accompany the lower concentration values, thereby, supporting the cause of lower concentration at that instant to be experimental errors. The general trend, however, makes us conclude that the short chain NPEs do not adsorb on glass container to any appreciable extent. Since, short chain NPEs were most hydrophobic of all the APEs tested, the glass containers may be safely used for storing the solutions for the test.

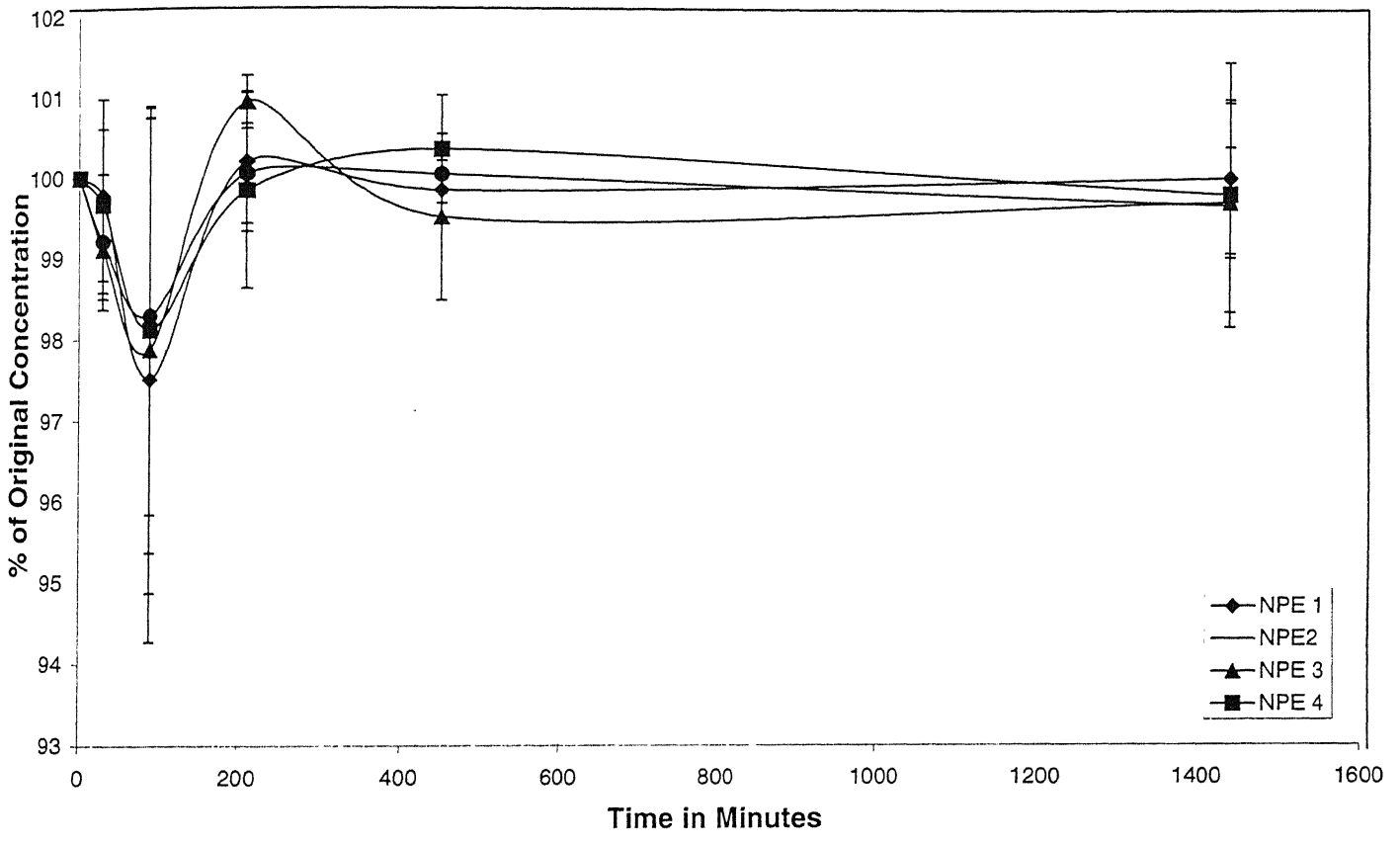


Figure 4.3. Adsorption of Short chain ethoxylates on glass surface with respect to time.

4.4.1.2. Test for Anaerobic Bacteria in Seed Solution for Anaerobic BFRs

The seeding solution for the Anaerobic BFRs was obtained from the denitrification tank of model Sewage treatment plant (STP) in the Engler Bunte Institute. It was decided to check the seed solution for the presence of anaerobes prior to seeding the reactors with it.

Anaerobic agar medium was prepared by dissolving the 51g of anaerobic agar medium in 1L of dematerialized water (demin) followed by boiling the solution for 15min. The solution was then sterilized by autoclaving at 121°C. The hot solution was poured in sterilized petri dish, which were then covered and kept to cool.

The plates were inoculated with the seed solution by pour plate technique and were incubated in an anaerobic airtight jar at 25°C for two days. Anaerobic conditions inside the jar were created using a gas pack. After two days of culturing in anaerobic conditions, good growth of bacterial colonies was observed on the plates, thereby, establishing the presence of anaerobes in the seed solutions for the anaerobic BFRs

4.4.1.3. Analyte Recovery with Sample Preparation

For any assay, in order to achieve appropriate results it is very important to have the sample in most readily analyzable form by the instrument. For this, prior to instrumental analyses, it is essential to perform the sample pretreatment. This ensures samples with appropriate analyte concentrations, well within the detectable range of the instrument. It also ensures that the analyte is present in matrix in which it can be analyzed with the instrument.

For concentrating the samples the process of Solid Phase Extraction (SPE) was used. The extracts were dried in the stream of Nitrogen (N2) followed by re-dissolution in Methanol.

For SPE, Waters Oasis™ HLB Extraction Cartridges, 6 cc (200mg), were used. These cartridges contain a copolymer with a designed Hydrophilic-Lipophilic Balance (HLB) to effect high recoveries. Teflon tubes were used to feed the sample volume of 100 mL to the cartridges. Care was taken to ensure that same tube was used for each reactor effluent and influent every time. The SPE was conducted as per the following steps:

- Cartridge conditioning with 5 mL Methanol.
- Cartridge Equilibration with 4 mL Water.
- Sample Loading: 100 mL of sample was extracted every time with one cartridge. The flow rate was maintained at 1-2 mL/min by adjusting the vacuum pressure.
- Sample container, Teflon tubes and cartridge washing with 5% Methanol.
- Drying of cartridge by application of vacuum.
- Elution with 2.5 mL of dichloromethane (CH_2Cl_2).

The eluents were dried in a steam of N_2 at 40°C. After drying the residue was dissolved in 100µL of methanol. To ensure the complete dissolution of the analytes in methanol, the vials containing the solution were kept in an ultrasonic bath for 5 min. Thus, the analytes were extracted from 100mL of SWW and were transferred to 100µL of methanol matrix. This helped in getting 1000 fold concentration of the analyte and also its presence in the organic solvent matrix, a prerequisite for HPTLC analyses. The analyte samples so prepared were then analyzed with HPTLC.

4.4.1.4. Recovery Tests

In order to determine the level of analyte recovery with the extraction procedure and to determine what concentration factor to use in the actual experiment, tests were carried out with known concentrations of analytes in the solutions being extracted. The tests were conducted individually for the three analytes (Marlophen NP3, Marlophen NP10 and Triton X-100), later to be used as test compounds in the biodegradation experiments. The stock solutions for all the three analytes were made in tap water and the dilutions were made in SWW, the matrix in which the analytes were fed to the reactors. Three replicates of spiked SWW samples were tested for each compound. The concentration levels of the analytes for three different compounds before and after SPE are listed in Table 2.4. The procedure followed was Solid Phase Extraction of analytes followed by the HPTLC Analyses of the extracts. The extraction of SWW sample (blank) was also included in the procedure as a method control.

Compound	Initial Concentration (mg/L)	Concentration factor	Final expected concentration (mg/L)
Marlophen NP10	5.0	1000	5000.0
Marlophen NP3	5.0	1000	5000.0
Triton X-100	5.0	1000	5000.0

Table 4.2. Concentration levels for the analytes before and after sample pretreatment

The recoveries obtained for considered ethoxylates in all the three analytes *viz.* Marlophen NP10, Triton X-100 and Marlophen NP3 can be seen in table 3.3, table 3.4 and table 3.5 respectively.

Sample	%Recoveries	CV%
NPE 11	99.21	7.75
NPE 9	97.99	7.62
NPE 6	88.61	5.25
NPE 5	85.7	6.36

Table 4.3. Ethoxylates in Marlophen NP10

Sample	%Recoveries	CV%
OPE 11	96.23	6.48
OPE 9	95.02	5.65
OPE 6	89.24	4.28
OPE 5	85.68	4

Table 4.4. . Ethoxylates in Triton X-100

Sample	%Recoveries	CV%
NPE 4	62.25	5.72
NPE 3	57.42	7.48
NPE 2	53.66	8.51
NPE 1	50.66	12.27

Table 4.5. Ethoxylates in Marlophen NP3

In general, the recoveries were found to decrease with the decreasing length of the ethoxylate chains. The recoveries for longer chain NPEs in Marlophen NP10 were in the range of 85 – 100 %. The long chain OPEs in Triton X-100 were also observed to follow the identical trends and showed a recovery in range of 85 – 100 %. However, the shorter chain NPEs in Marlophen NP3 were recovered much less after sample pretreatment. Their recoveries ranged from 50 – 65 %. Thus, it was concluded that there is no significant loss of long chain ethoxylate analytes in the pretreatment process and hence it was not needed to apply the recovery factor for Marlophen NP10 and Triton X-100 in the experiments. However, the recoveries of shorter chain ethoxylates were fairly low and so

it was decided to apply a recovery factor of 60%, in case any short chain ethoxylate were found to emerge as degradation products in the experiments.

4.4.2. Development of Biofilm Reactors

4.4.2.1. The Biofilm Reactors

The miniaturized biofilm reactors consist of a stainless steel shell inside which is placed a steel mesh mounted on plastic supports. Within the mesh is placed the media (pumice stone) on which the biofilm grows. The reactors are placed upright and the flow through them is maintained against gravity.

Eight biofilm reactors were setup in all. Of them, 3 aerobic and 3 anaerobic reactors were setup to feed the three different surfactants individually to one aerobic and one anaerobic BFR. One aerobic and one anaerobic reactor were fed only by SWW (control). A schematic of the experimental logistics is shown in Figure 4.4.

4.4.2.2. Setting up the BFRs

Plastic vials with steel mesh were filled with pumice to form the support media for the growth of biofilms. These vials were placed inside the steel shells forming the body of reactors (Figure 4.5). The reactors were placed upright. Both inlet and outlet channels were connected to Teflon tubes. A flow of 1mL/min was maintained by means of a pump. The tubings used on the pump were Tygon 1.85mm i.d.(for liquid inlet) and Tygon 0.95 mm i.d.(for air inlet). The airflow through the aerobic reactors was ensured by coupling the liquid inlet and air inlet tubes together before they entered the Teflon tube which carried the influent to the reactor. (Figure 4.6)

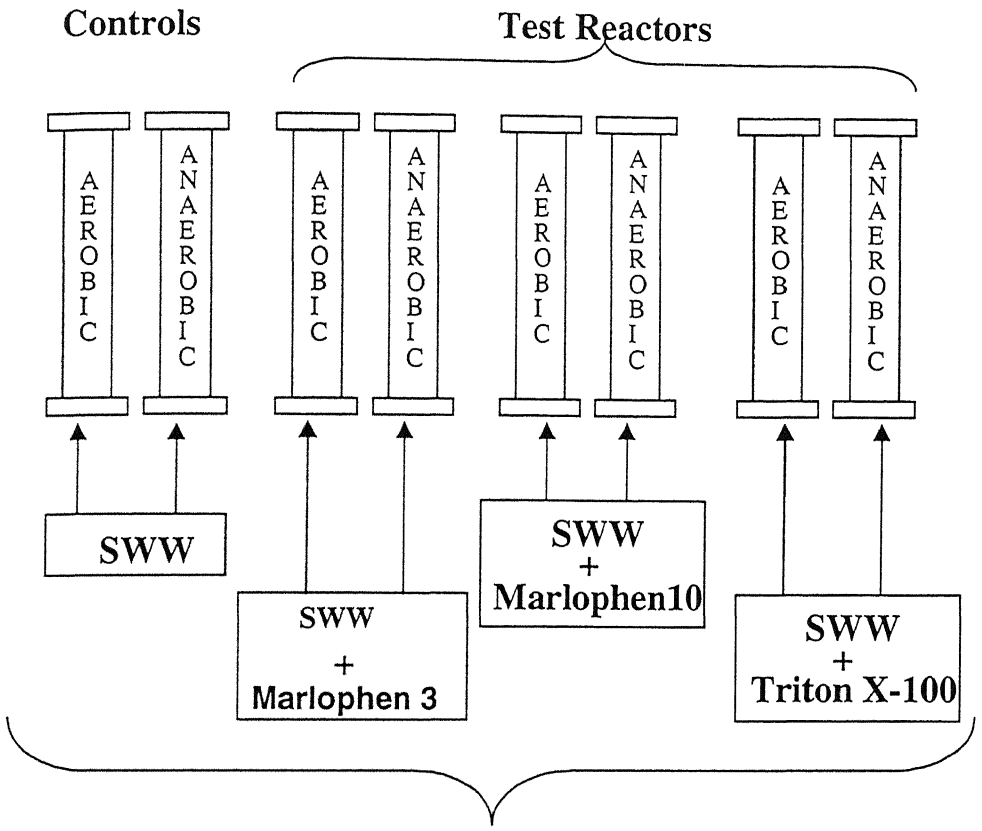


Figure 4.4. Setup of biofilm reactors.

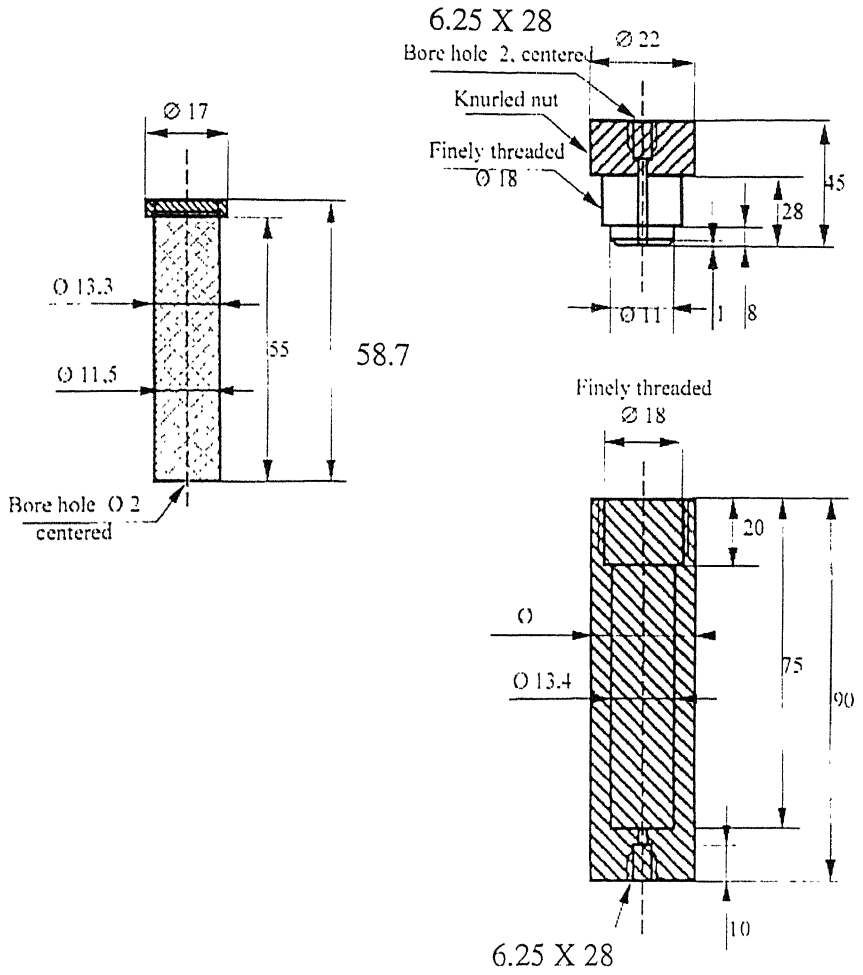
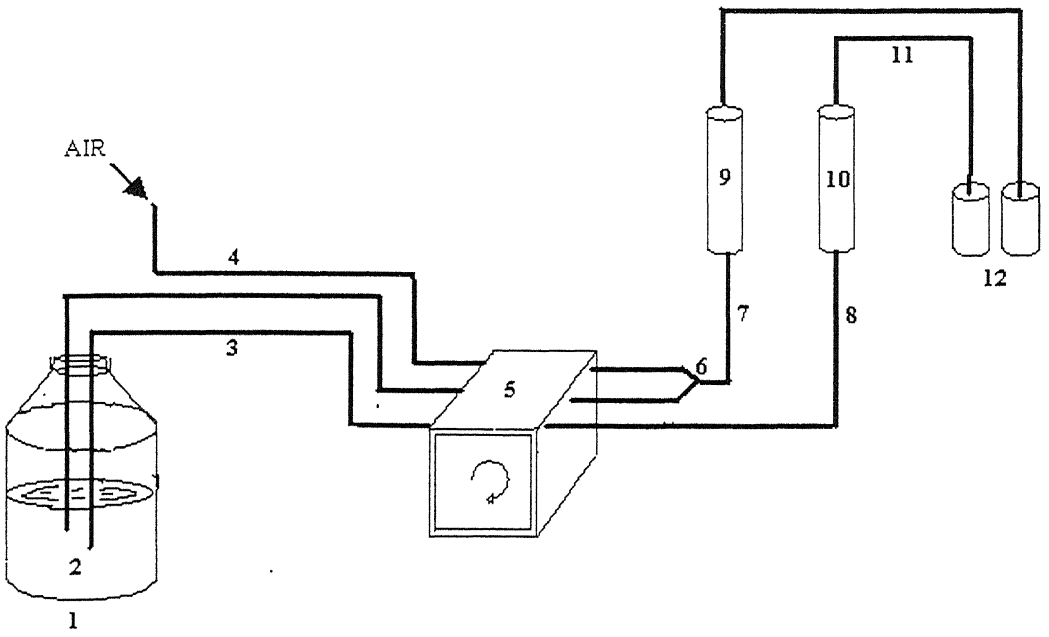


Figure 4.5. Structural details of a Biofilm Reactor.



1 => Influent Container.
2 => SWW + Test Compound (Test feed).
3 => SWW Influent pipe from container to pump.
4 => Air inlet pipe to pump.
5 => Pump maintaining a flow rate of 1mL/min.
6 => T-junction for mixing air and test feed for Aerobic reactor.
7 => Influent pipe from T-Junction to Aerobic reactor.
8 => Influent pipe from Pump to Anaerobic reactor.
9 => Aerobic reactor.
10 => Anaerobic reactor.
11 => Effluent pipes from reactors.
12 => Effluent collection containers.

Figure 4.6. Schematic representation of experimental set up.

4.4.2.3. Colonizing the BFRs

To colonize the aerobic BFRs, a mixed bacterial population was obtained from the activated sludge tank of the Neureut wastewater treatment plant near Karlsruhe, Germany. The activated sludge was settled and decanted. Three liters of the decant was diluted with tap water to 10 L in a plastic container. To colonize the anaerobic BFRs, the seed was taken from the anaerobic denitrification tank of a model STP in Engler-Bunte Institute, Karlsruhe, Germany. 3L of the mixed bacterial culture solution from the denitrification tank was diluted with tap water to 10 L in another plastic container. These solutions were then fed to their respective aerobic and anaerobic reactors in a cyclic fashion for a week. Everyday, concentrates for SWW were added to the solution. After a week the cyclic flow was stopped and the fresh SWW solution without any seed was allowed to flow through the reactors.

4.4.2.4. Daily Maintenance

Everyday (between 9 and 10 am) fresh SWW was prepared as described in figure 3.2. This was fed to the reactors continually. The plastic container which contained SWW was cleaned everyday with 35% H_2O_2. The tubes (Teflon and pump tubings) were cleaned likewise once a week. To analyse the performance of reactors with respect to time of conditioning, DOC measurements for the influent and the effluent were made on Shimadzu DOC Analyser at regular intervals of time.

4.4.2.5. DOC Measurement

Dissolved organic matter (DOM) provides energy and nutrients for heterotrophic bacteria in aqueous systems (resulting in DOM degradation by bacteria), but not all DOM can be metabolised. The chemical complexity of DOM complicates the analytical task of identifying each molecule (composing the DOM) and estimating individual rates of degradation. Alternatively, dissolved organic carbon (DOC), comprising 40–50 % of the elemental composition of DOM has been used as an analytical surrogate to measure DOM degradation (Kaplan, 1995). Concentrations of biodegradable DOC (BDOC) can be estimated from changes in DOC concentrations following exposure of water samples to the microorganisms. In case of BFRs, the difference between the DOC concentrations between the reactor inflow and outflow and outflow provides the estimate of BDOC. The determination of the DOC contents of the samples was done on Shimadzu Total Organic Carbon Analyser (Model TOC-5050).

4.4.2.6. Monitoring Reactor performance by Measuring DOC

The collected influent and effluent samples were filtered through 0.45µm HTTP filters (25mm diameter) before being analyzed on Shimadzu TOC analyzer. Two replicates per sample were measured every time. Every effort was made to analyze the samples directly after collection. In cases, when this was not possible, the samples were frozen and analyzed at a later date.

4.4.3. Investigations on Biodegradation of APEs

The HPTLC coupled with AMD was used as the method of separating individual ethoxylates in the compound mixture. Fluorescence detection was used as the detection method of the separated analytes. The entire analyses was performed as per the following steps:

4.4.3.1. Application of analytes to the test systems

The analysis for biodegradation of APEs was started after about 10 weeks of conditioning of the reactors with SWW. Marlophen NP3 was used as the source of short chain Nonylphenol ethoxylates, Marlophen NP10 was used as source of long chain NPEs and Triton X-100 was used for providing Octylphenol ethoxylates in the test system.

The stock solutions (250 mg/L) of Marlophen NP10 and Triton X-100 were prepared in dematerialized water (demin) every five days while stock solution (50 mg/L, not a true solution but when diluted further to 5mg/L results in a true solution) of Marlophen NP3 was made in demin every second day. Subsequent dilutions from these stock solutions were made everyday using SWW so as to finally prepare 5L solution of each of these compounds having compound concentration to be 5 mg/L. The solutions so prepared were then fed respectively to the aerobic and anaerobic reactors designated as test reactors for the particular analyte. However, the control reactors were fed by only the SWW, without spiking it with any of the test compounds. The 5L glass containers already analyzed for the amount of analyte adsorption on them (section 3.4.1.1), were used as containers for the test solutions. Fresh analyte and control feed solutions were prepared every morning and fed to the reactors between 9.30 and 10 am. Care was taken

that the same container was used for a particular analyte and also for control solution everyday. The containers were cleaned everyday with 35% H_2O_2 and the tubings were cleaned likewise twice a week.

4.4.3.2. Sampling

The influents as well as effluents from all the eight BFRs were sampled thrice a week. The samples for TOC measurement (25 to 30mL) were collected approximately 2 to 2.5 hours after preparation of analyte and control feed solutions in the morning. Samples for analyses with HPTLC (100mL) were collected immediately after sampling for TOC measurement. Care was taken that same flasks were used to collect effluents from a particular reactor in every sampling turn.

The collected samples were filtered through a 0.45 μm filter (25 mm diameter) using a 20mL syringe. The syringe was washed after every filtering step with Milli Q so as to ensure no contamination of one analyte sample with another. Two replicates per sample were tested with the Shimadzu TOC Analyzer.

The influent and the effluent samples were extracted by the solid phase extraction method. Elution was done using Dichloromethane and the extracts were then dried under a stream of N_2 at 40°C. The residues were redissolved in 100μL of Methanol, thereby ensuring 1000 fold concentration of analyte and also a change from aqueous matrix to organic solvent matrix.

4.4.3.5. Preconditioning of Silica Gel Plates

Merck, silica gel 60, HPTLC precoated plates, 10 x 20cm, without fluorescence indicator, were used as the stationary phase for HPTLC. Prior to sample application, the plates were developed with 2 – propanol (Chromatography grade) and left in the isopropanol solution overnight. The plates were then activated for 30 min in a preheated oven (110°C). The plates were then covered with a clean glass cover and stored in a desiccator until their usage.

4.4.3.6. Sample Application

Sample application was performed by the spray on technique using Automatic TLC Sampler III, Camag, Switzerland. 2μL of all samples and standards were loaded as separate bands on the silica gel plates. All the bands were 5mm long and were separated by a distance of 8mm from center to center of adjacent bands. The bands were laid in duplicate for each sample on one silica gel plate. The sample application speed of the instrument was adjusted at 200 nL/s. Along with all the samples, a series of analyte standards of concentrations 2, 3, 4 and 5 g/L (2 μL each) were also applied on each chromatographic plate.

4.4.3.7. Chromatogram Development

The chromatograms were developed using Automated Multiple Development (AMD). The HPTLC plates were developed by gradient elution in the AMD system. In an AMD system, the HPTLC plate is subjected to stepwise gradient development in an enclosed

chamber. The developing solvent mixture is prepared in a gradient mixer from the solvent components in separate bottles. Between developments, the mobile phase is removed from the developing chamber and the plate is dried under vacuum and then conditioned in an atmosphere of controlled composition.

The Camag AMD 2 instrument was used for chromatogram development. A 22 step, gradient elution method, developed at Engler-Bunte Institute, Karlsruhe, on the basis of method suggested by Jun et. al. (1998) incorporating alterations to make the method also effective for the separation of non-polar, short chain ethoxylates, was used to develop the chromatograms. The solvents used for development were ethyl acetate and water mixture (12:0.5), acetone and n-hexane. At every step the ratio of the three solvents with respect to each other was varied so that a gradient from polar to non-polar characteristics of the solvent could be achieved.

The detailed description of solvent composition in individual AMD steps can be seen tabulated in the appendix I. Very good separation between the component ethoxylates in the mixture of APEs was obtained with the help of HPTLC/AMD for all the three compounds under analyses.(Figure4.7 a, b, and c)

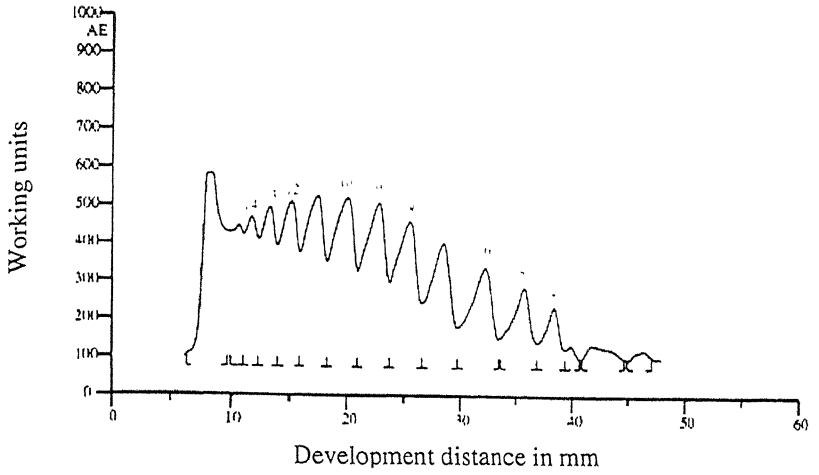


Figure 4.7. a. Separated Nonylphenol ethoxylates with different ethoxylate chain lengths in mixture of long chain NPEs in Marlophen NP3.(Std Marlophen NP3, 5g/L)

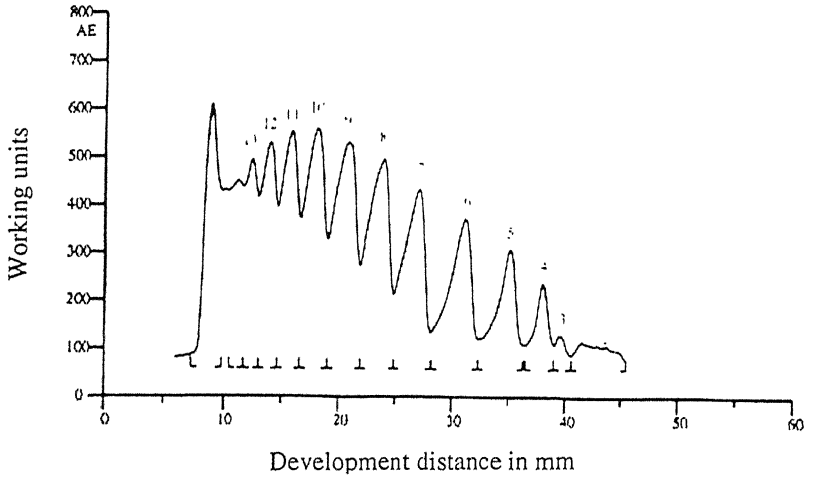


Figure 4.7. b. Separated Octylphenol ethoxylates with different ethoxylate chain lengths in mixture of long chain OPEs in Triton X-100.(Std Triton X-100, 5g/L)

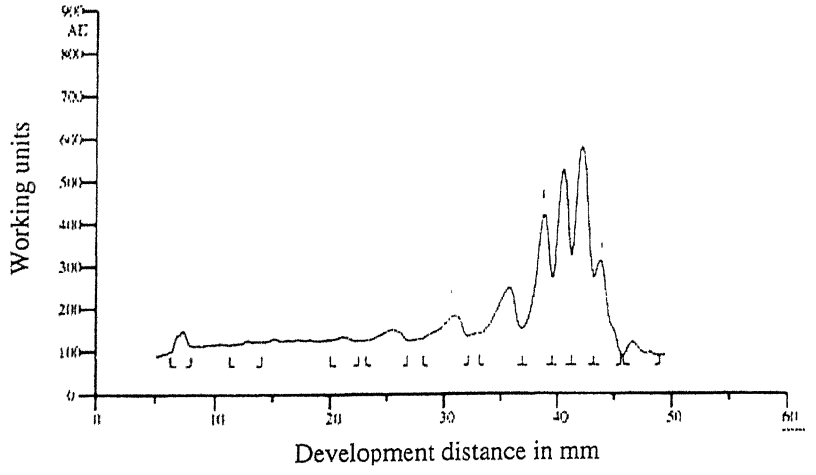


Figure 4.7. c. Separated Nonylphenol ethoxylates with different ethoxylate chain lengths in mixture of short chain NPEs in Marlophen NP3

4.4.3.8. Fluorescence Signal Enhancement

The plates developed with AMD were dipped in a solution of Paraffin and Hexane (1:2) using Camag Chromatogram Immersion Device III [DIN 38407-7]. The plate was kept in the solution for 2 s and was moved in and out of the solution chamber at a constant speed of 2.5cm/s. After dipping, the plate was allowed to dry. This process of coating the silica gel plate with a layer of paraffin helped in increasing the fluorescence, thereby, facilitating the better detection by obtaining larger peaks in the chromatograms.

4.4.3.9. Densitometric Evaluation

The developed plates were scanned with TLC scanner 3,(Camag, Muttenz, Switzerland) using CATS evaluation software. Fluorescence detection was used at an excitation wavelength of 227nm. The zero position for the instrument calibration was set manually at some place on the plate where no substance could possibly reach (a blank band was introduced most often but if this was not possible due to excess of analytical substances, the zero position was set at some place below the line of sample application on the Silica gel plates). The sensitivity of the instrument was varied with every measurement (a function of chromatogram development) to optimize the signal detection. However, the sensitivity used for longer chain APEs was in range of 125-140 while that of short chain ethoxylates was in range of 95-110. A Deuterium lamp was used to induce the fluorescence and the emission was measured using a cut-off filter of wavelength $\leq$ 280nm. The slit dimensions used were 4 x 0.45mm and a multicolor plotter was used for graphic presentation of the obtained chromatograms.

4.4.3.11. Tests for Carboxylate Derivatives of the Analytes

Since the alkylphenol carboxyethoxylates are one of the probable end products of the biodegradation of APEs, tests were conducted to check for their presence in the effluents of biofilm reactors. The influent and the effluent samples after being extracted once (for APEs and AP) were collected in the glass vials. These samples were then acidified using HCl to a pH in range of 2 – 2.5. These samples were again extracted using new SPE cartridges. The eluents were dried and residues were then analyzed by HPTLC for presence of any new compound.

4.4.4. Investigations on Cell Activity and the Types of Bacteria in Biofilms

4.4.4.1. Determination of Types of Bacteria in Biofilms

To find the type of bacteria present in large numbers in the biofilms of water and wastewater treatment plants has been a question of interest to most of the microbiologists and environmentalists. Efforts were made to characterize the dominating classes of bacteria present in the biofilms by performing Fluorescent *In situ* Hybridization (FISH) analysis of the biofilm samples from the reactors.

The biofilm reactors were sampled twice at different intervals of time for their FISH analysis – once before feeding the analyte to the BFRs and a second time when the reactors had well acclimated to the analyte substance (as could be monitored by DOC degradation and HPTLC analysis of their effluents). The reactors were dismantled and the plastic vials containing pumice and biofilms were taken out from the steel shells.

About 5mg of biofilm was scrapped of from all the sides of the vials ensuring uniform sampling, using a sterilized steel scalpel. The biofilm was then mixed in an isotonic solution of sodium chloride (NaCl) and vortexed to form a uniform suspension. Sampling was done from the control (both aerobic and anaerobic) reactors before feeding the analyte to the reactors and from control as well as test reactors for biodegradation of Marlophen NP10 (both aerobic and anaerobic) after feeding the analyte. The samples were then handed over to the experts in handling FISH, in Engler-Bunte Institute, Karlsruhe. The bacteria were analyzed with probes for Eubacteria and Archeabacteria. The eubacteria were further analyzed for phylum Proteobacteria that is the largest (containing 441 genera) of all the 23 phyla of Eubacteria (Garrity et al., 2001). Further Proteobacteria were also analyzed for its 3 different classes (out of 5 classes) *viz* Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria, all three of them being Gram negative bacterial classes. However, analysis could not be done for other two classes *viz.* Deltaproteobacteria and Epsilonproteobacteria due to non-availability of probes. The slides were observed using an epifluorescence microscope (Leica/Leitz LaboLuxS). The details of the cut off filters used for analysis are listed in table 4.6

Filter Label	Excitation Light	Excitation Light Filter	Emission Filter	Emission Filter	Applicable to Dye
A 4	UV	BP 360/40*	400	BP 470/40	DAPI
N 3	Green	BP 546/12	565	BP 600/40	Cy3, CTC
L 5	Blue	BP 480/40	505	BP 527/30	FLUOS
Y 5	Red	BP 620/60	660	BP 700/75	Cy5

Table 4.6. FISH probes used for analyses of bacteria types
(BP = Band Pass Filter)
* a/b ; a = Excitation wavelength in nm, b = ± band range)

4.4.4.2. Determination of cell activity in BFRs

The samples collected from control and test reactors were also analyzed for the proportion of active cells as compared to whole cell number. This was done by using Millipore Membrane Filters (0.2μm, GTBP) through which the samples were filtered and the cells were fixed on membranes. DAPI (4,6- Diamidino 2-phenyl-indol-dihydrochloride), a DNA binding dye was used to characterize the whole cell number, while CTC (5-Cyano 2,3,dimethyltetrazolium chloride), an oxidizing compound was used to determine the active cells and thus the cell activity in the reactors was determined.

4.4.5. Investigations on Analyte Removal by Adsorption onto the Biofilm

Alkylphenol Ethoxylates and their metabolites have a lipophilic moiety that makes their adsorption onto the surface of biofilms fairly likely. To ascertain the extent of removal of APEs and their metabolites due to adsorption onto the biofilms, efforts were made to recover the analyte and its metabolites from the surface of biofilms.

4.4.5.1. Determination of Weight of Biomass formed

The weight of biomass formed was determined as the difference in the weight of the reactors before and after the experiment. Prior to the colonization of the reactors (at the time of setting up), the vials containing the pumice stone were heated at 105°C for 24 hours. These vials with pumice after heating, the steel shells of the reactors and the connective tubes for the reactors were weighed. At the end of the experiment, the influent supply to the reactors was stopped and the reactors were dismantled. The vials

(containing the pumice stone and the biofilm) were removed from the reactors and dried at 105°C for 24 hours. These vials with pumice and biofilm, the steel shells and connective tubes were weighed again. The difference in weight of the vials before and after the experiment gave the weight of the biomass.

4.4.5.2 Extraction of the Analyte and Degradation Products Adsorbed onto the Biofilms

Two extractions were carried out serially, with hexane and with methanol. This was done to ensure that both the polar and the non-polar analytes could be extracted from the biofilms.

4.4.5.2.1. Extraction with Hexane

The dried pumice stone and the biomass were transferred to a glass vial and covered with about 20mL Hexane. The vials were then put on a rotator for 2 hours to ensure contact between the biomass and the solvent. Later, the liquid phase was decanted in another vial. The remaining biomass and pumice stones were washed further with 5mL hexane and the washings were added to the decant. The decanted solution was centrifuged for 8min at 2,500 r.p.m. The clear solution so obtained was dried in a stream of N_2 and the residue in the vial was redissolved in 200μL methanol. The solution thus obtained was analyzed with HPTLC.

4.4.5.2.2. Extraction with methanol

The biomass and pumice stone left after extraction with hexane were extracted with 20 mL Methanol. The procedure followed was the same as for hexane extraction. The solutions so obtained were also analyzed with HPTLC.

Chapter 5

Results and Discussion

5.1. Reactor Development

During the period of reactor development, both aerobic and anaerobic reactors were fed with the same feed but with different seed sludge, as outlined in section 4.4.2. The DOC removal in the reactors was monitored at regular interval to assess the reactor performance. The DOC serves as food for microorganisms in the biofilms. Therefore, higher DOC depletion is indicative of effective biofilm development in the reactors. The DOC removal in both aerobic and anaerobic reactors is shown in Figure 5.1.

At the start, the reactors maintained under aerobic condition showed higher DOC removal as compared to the anaerobic BFRs. Both, aerobic and anaerobic BFRs showed rapid colonization during the initial two weeks of operation. During this period, the DOC removal increased by about 25% in both aerobic and anaerobic BFRs. Thereafter, the performance of the aerobic BFR remained in the range of 80-85% removal of DOC. A slight decrease in the performance of the anaerobic BFRs was observed between 25th and 45th days of operation. However, both the reactors stabilized and reached a steady state under the operating condition by the 60th day of operation. The steady state DOC removal in all the BFRs was between 80 – 85 %. Another ten days were allowed for the conditioning of the reactors. The target compounds (APEs) were applied to the reactor starting from the day 73.(which corresponds to day 0 of Degradation test phase).

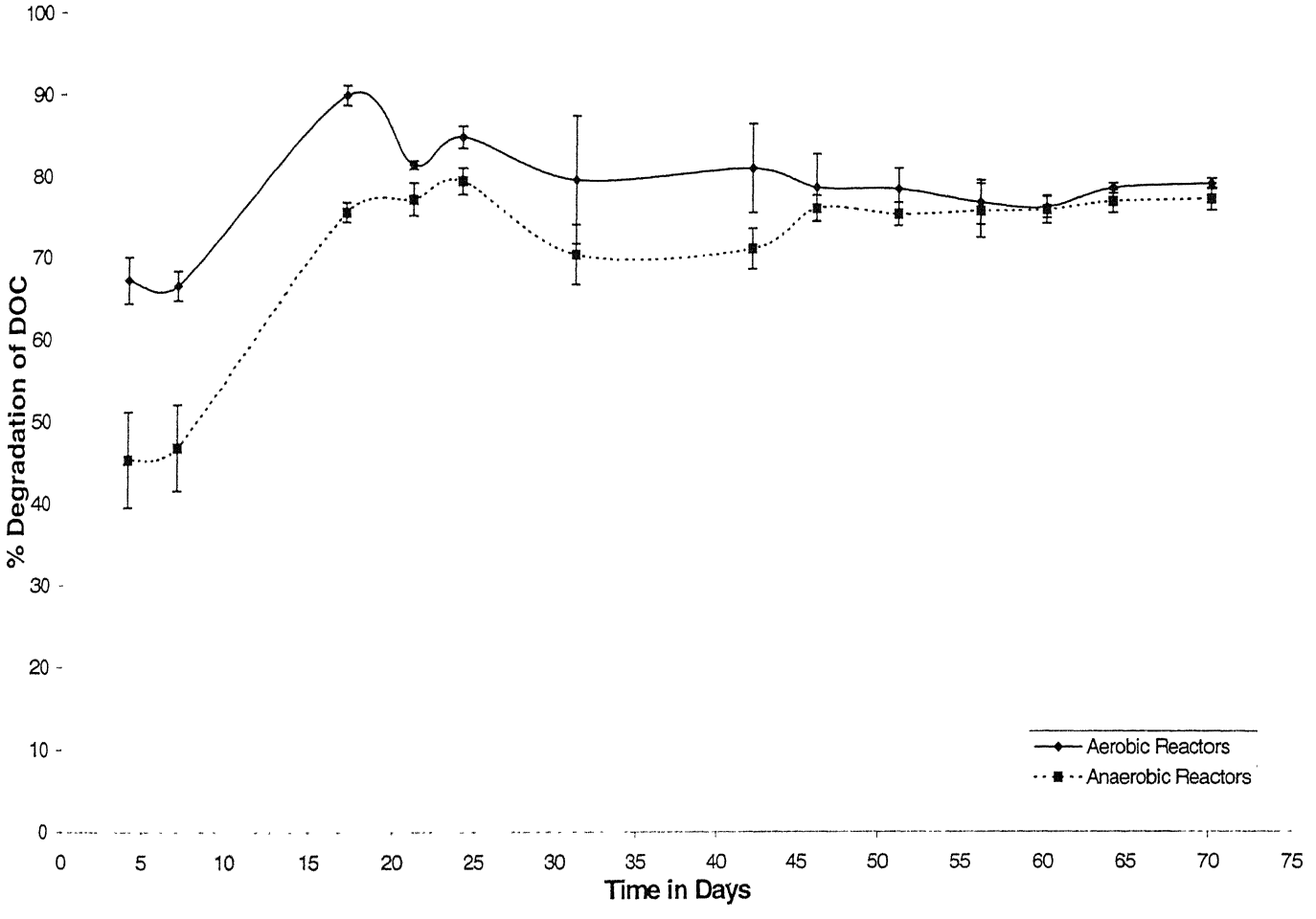


Figure 5.1. Average % reduction in DOC by aerobic and anaerobic reactors during colonization phase

5.2. Degradation of Alkyl-Phenol Ethoxylates (APEs)

The addition of the APEs in the influents to the test reactors increased the DOC of the influent by about 15%. In the control reactors, the influent DOC remained same as that used during the development of the reactors. The contribution of each APE compound to the DOC of influent is shown in table 5.1.

DOC Contributor	DOC (g/L), Mean ± Std. Deviation
DOC of SWW matrix	10.664 ± 0.441
DOC of Marlophen NP10	1.727 ± 0.639
DOC of Marlophen NP3	1.582 ± 0.635
DOC of Triton X-100	1.807 ± 0.636

Table 5.1. DOC of the feed solution and the APE additives in the experiments.

The overall biodegradation in the reactors was monitored by periodic DOC analysis of the influent and effluent. The degradation of target APE compounds was monitored by the HPTLC analysis of the influent and the effluent samples at regular intervals of time. Three APE compositions tested were, a mixture of Octyl-phenol Ethoxylates commercially available as Triton X-100, a mixture of short-chain Nonyl-phenol Ethoxylates commercially available as Marlophene NP3 and a mixture of long-chain Nonyl-phenol Ethoxylates commercially available as Marlophene NP10. These are mixtures of molecules with different number of ethoxylate units. The biodegradation results are reported and discussed in the following sections.

5.2.1. Biodegradation of Octyl-Phenol Ethoxylates

Biodegradation results of Triton X 100 in aerobic BFR and in anaerobic BFR are shown the figures 5.2 and 5.3, respectively. The DOC reduction in the control reactors remained constant (80-85%) as was attained at the end of the reactor development phase. The addition of the OPEs instantly reduced the overall DOC removal by about 60% although the contribution of OPE to overall DOC was only 15%. The overall DOC removal gradually increased to about 75% in two weeks time and continued at that level for the rest of the duration of the experiments (50 days). Although, apparently this 75% reduction in influent DOC at steady state is a decrease compared to 80-85% found in the control reactor, the total amount of organic carbon removal remained same as the influent DOC of the test reactors are 15% more than that of the control. These observations are similar for both aerobic as well as the anaerobic BFRs.

The initial acclimation phase of two weeks could not be attributed to enzyme induction alone or low population density. The bacterial population was already acclimatized to the basal media that supplied 85% of the DOC in the test reactors. There was also sufficient number to degrade this basal media by about 85%. The acclimation phase may be due to any of the following reasons or their combination: (i) the addition of OPEs inhibited the degradation of substrates present in the base media until the bacteria started producing enzymes to degrade OPEs and reduce their concentration; (ii) OPEs were toxic to certain species in the population. Some species were capable of degrading the OPEs took 2 weeks to grow in sufficient number and as a result, the population composition shifted

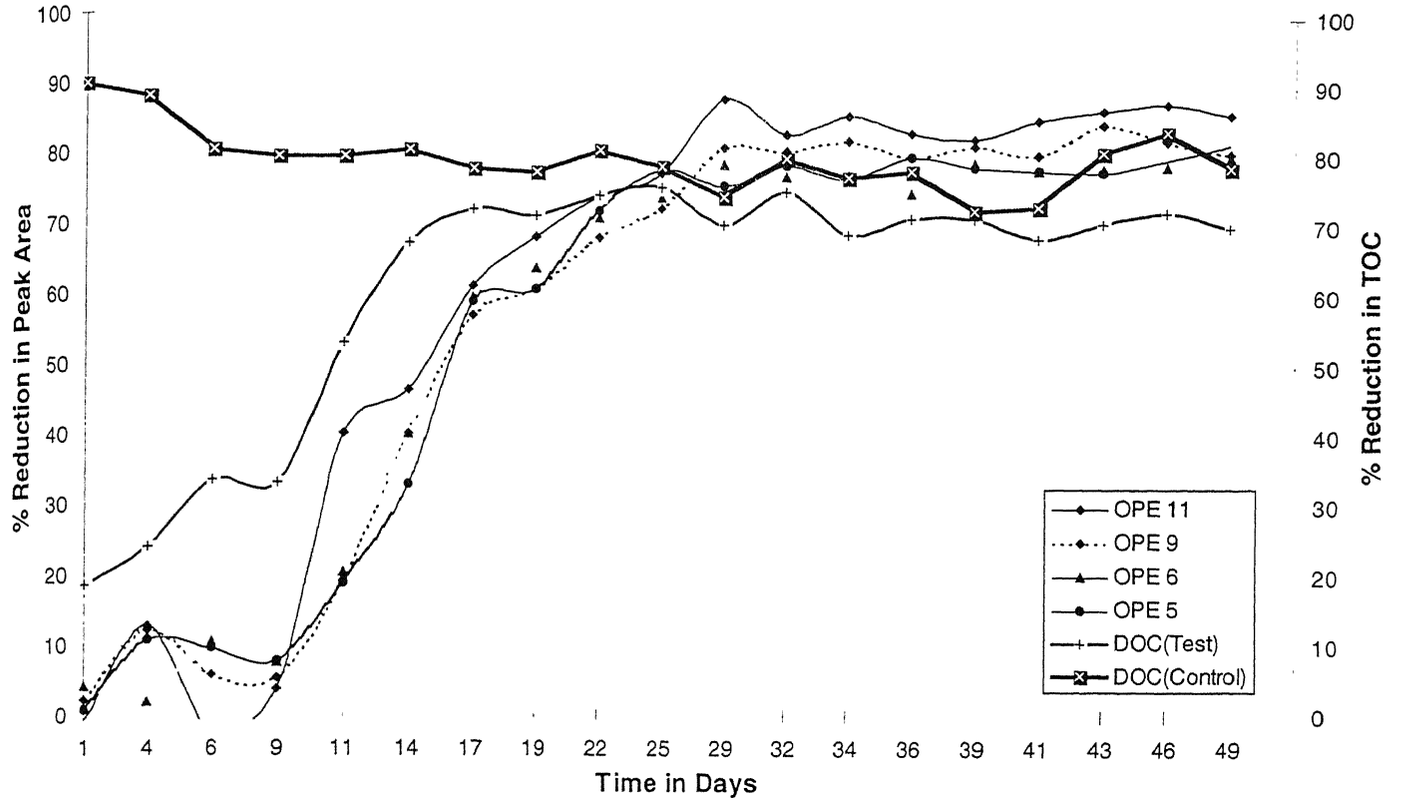


Figure 5.2. Degradation Curves for different long chain OPEs in aerobic BFR fed with mixture of long chain OPE.

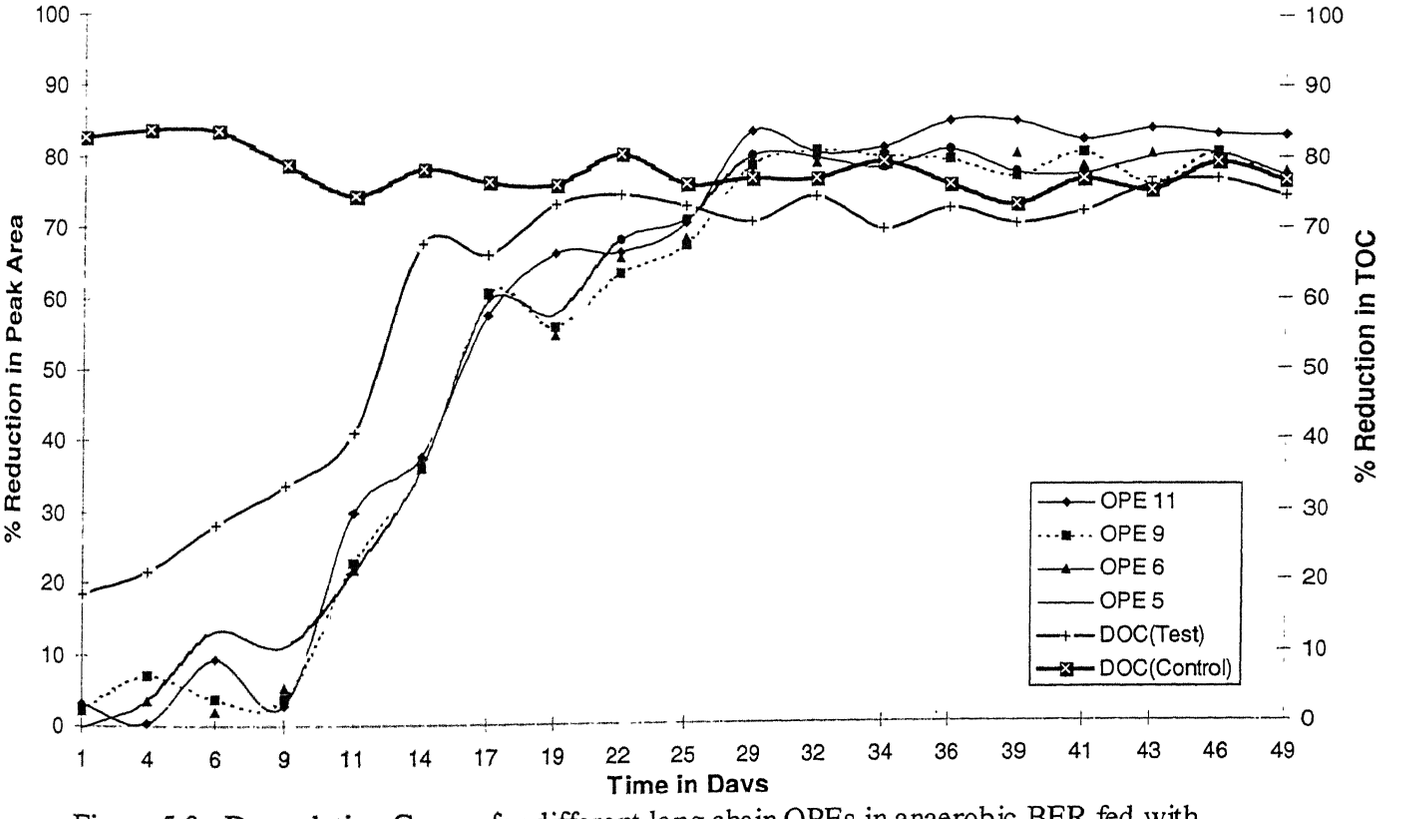


Figure 5.3. Degradation Curves for different long chain OPEs in anaerobic BFR fed with mixture of long chain OPEs.

With the help of HPTLC analysis, it was possible to monitor the biodegradation behavior of individual ethoxylates in the mixture. For performing the degradation analysis of the OPEs, four different ethoxylates viz. OPE 11, OPE 9, OPE 6 and OPE 5 were selected. The general trends in the biodegradation pattern for OPEs were observed to be fairly identical showing a variation of ± 5% at any instant of time among each other (Figure 5.2 and Figure 5.3). Both the aerobic and anaerobic BFR exhibited a similar lag phase. The lag was followed by an increasing rate of degradation of the target compounds until the removal were stabilized at about 80 – 85%, both in aerobic and anaerobic reactors. However, the following points may be noted: (i) the total time taken to reach the steady state of OPE degradation was about 30 days as compared to that taken for overall DOC removal was only 14 days; (ii) the removal of OPEs at steady state is about 10% more than the overall DOC removal. These two observations suggest that, once the population is well stabilized, the OPE becomes the preferred substrate whereas during the acclimation phase of first 25-30 days, the basal media was the preferred substrate.

5.2.2. Degradation of Long Chain Nonyl-Phenol Ethoxylates

Biodegradation results of long chain NPEs in Marlophen NP-10 in aerobic BFR and in anaerobic BFR are shown in the figures 5.4 and 5.5, respectively. In addition to overall degradation analysis by DOC, four different target ethoxylate compounds viz. Nonylphenol Ethoxylate 11 (NPE 11), Nonylphenol Ethoxylate 9 (NPE 9), Nonylphenol Ethoxylate 6 (NPE 6) and Nonylphenol Ethoxylate 5 (NPE 5), were selected for HPTLC analysis. The overall degradation as well as the degradation of target compounds was observed to be similar to those of OPEs (section 4.2.1), for both aerobic and anaerobic BFRs. It may be noted, that molecules in Triton X-100 and Marlophen NP-10 have very

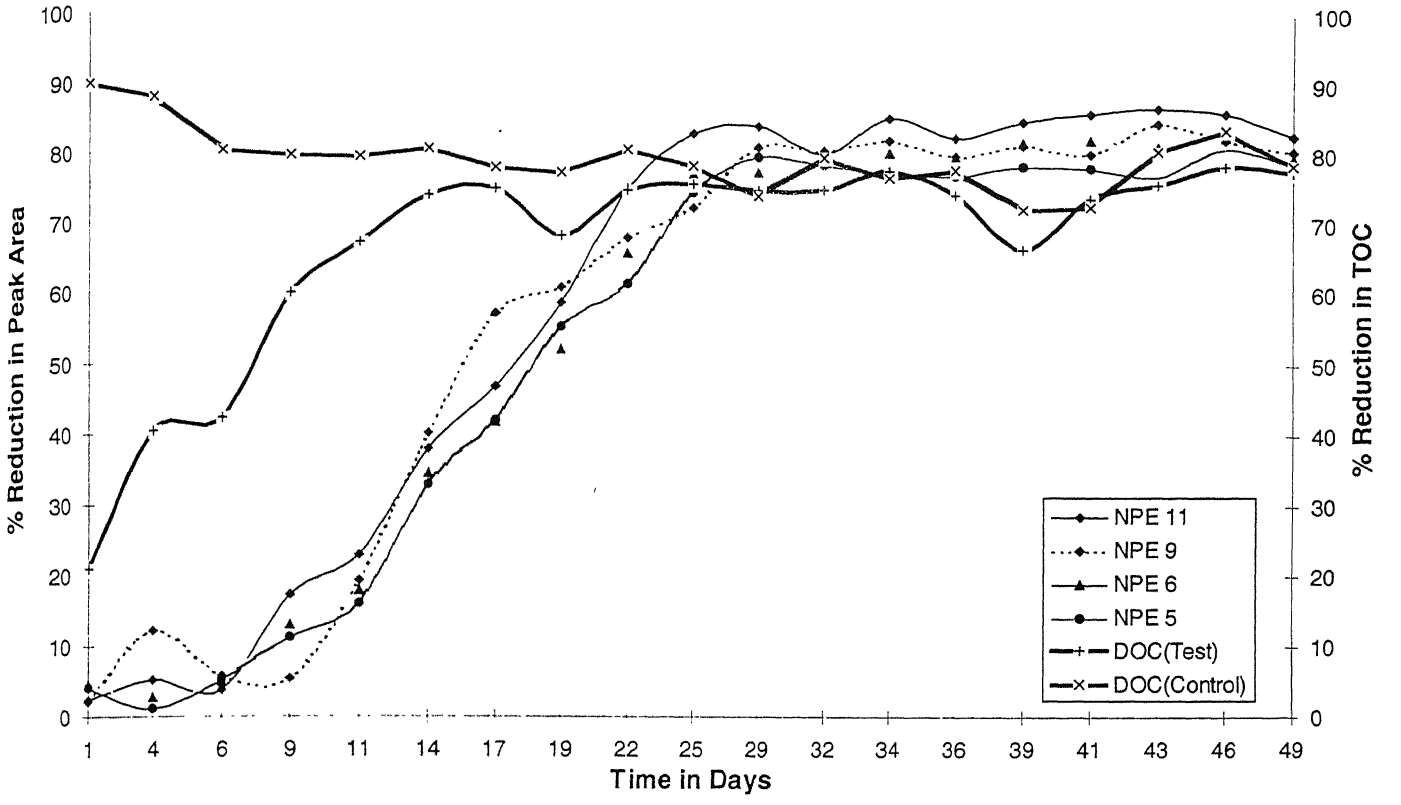


Figure 5.4. Degradation Curves for different long chain NPEs in aerobic BFR fed with mixture of long chain NPE

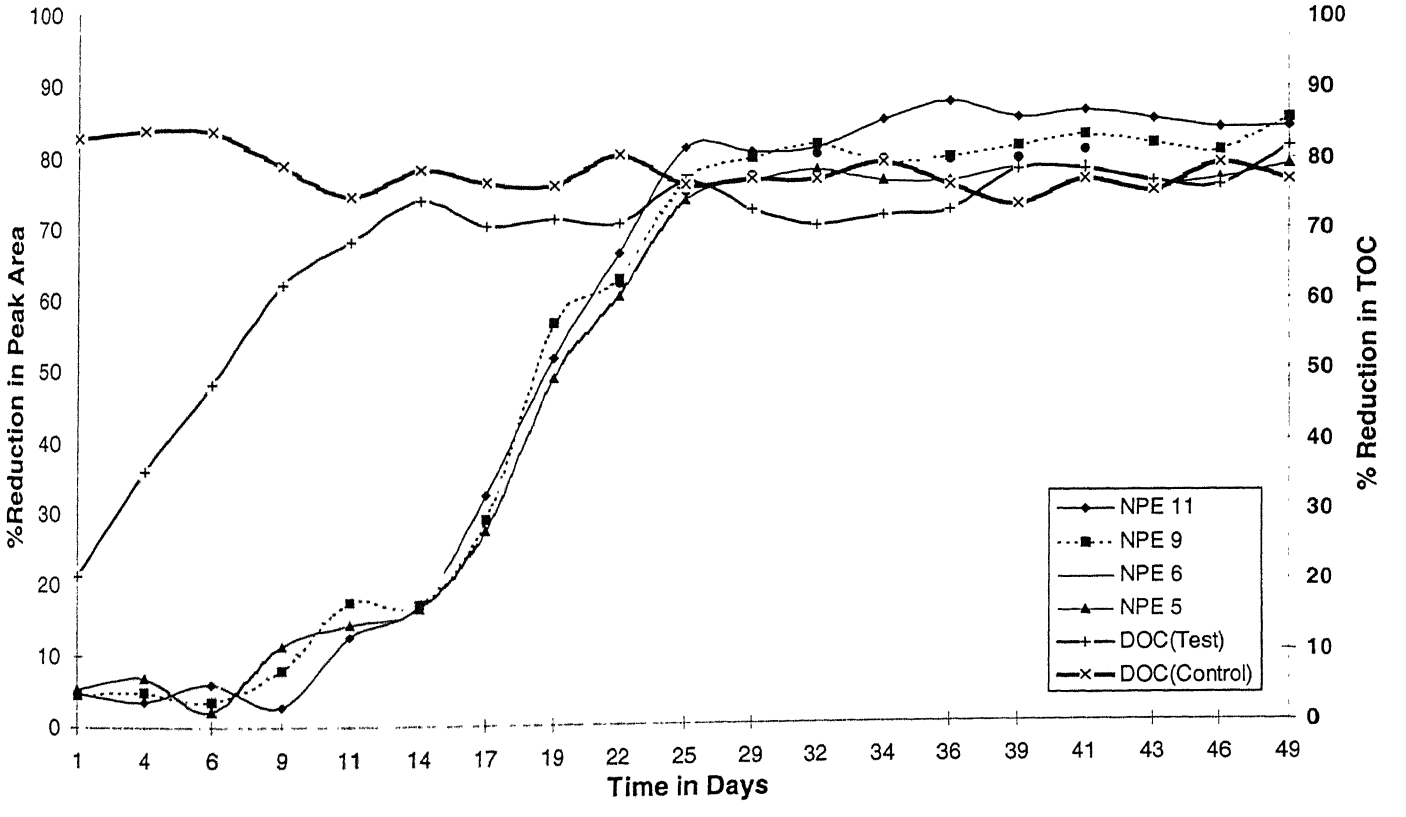


Figure 5.5. Degradation Curves for different long chain NPEs in anaerobic BFR fed with mixture of long chain NPE

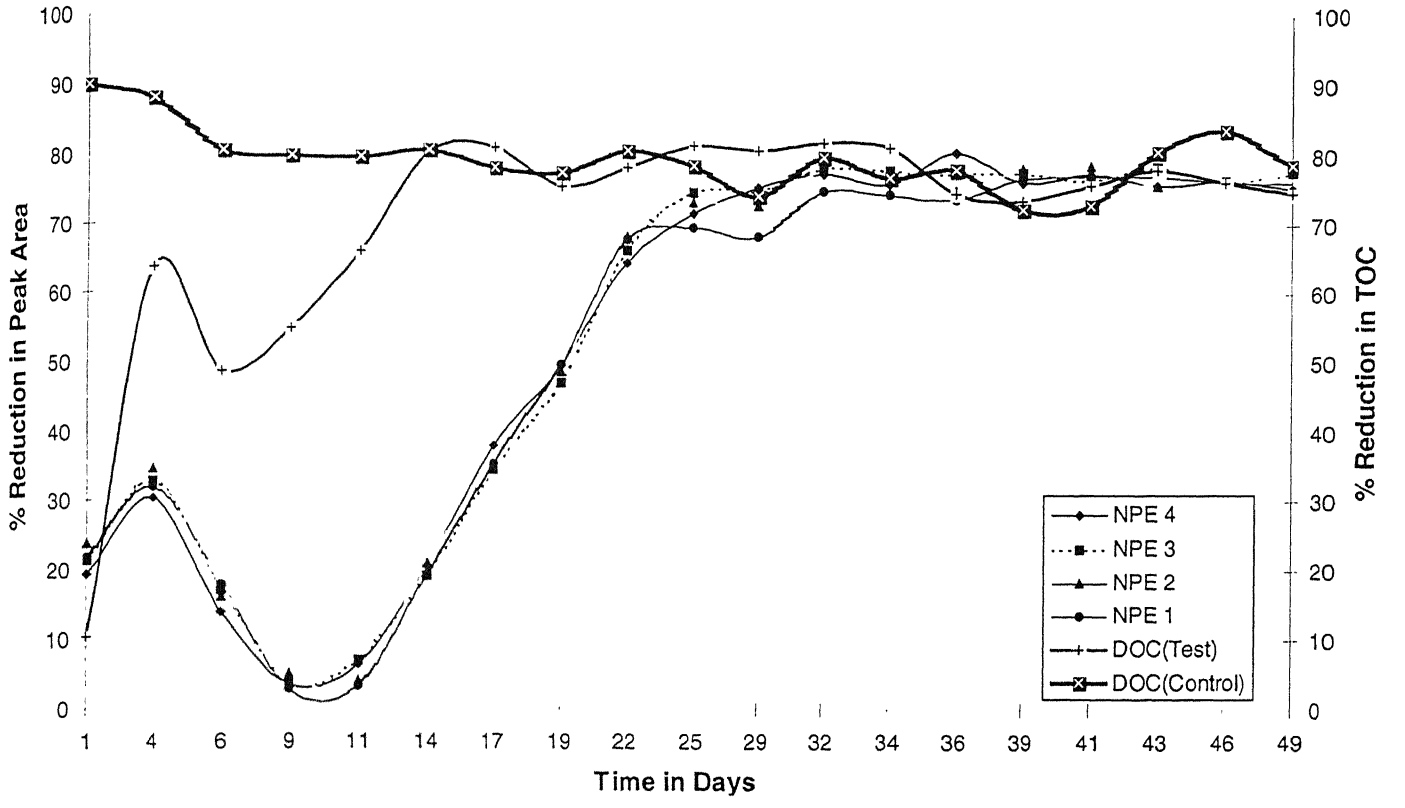


Figure 5.6. Degradation Curves for different short chain NPEs in aerobic BFR fed with mixture of short chain NPEs.

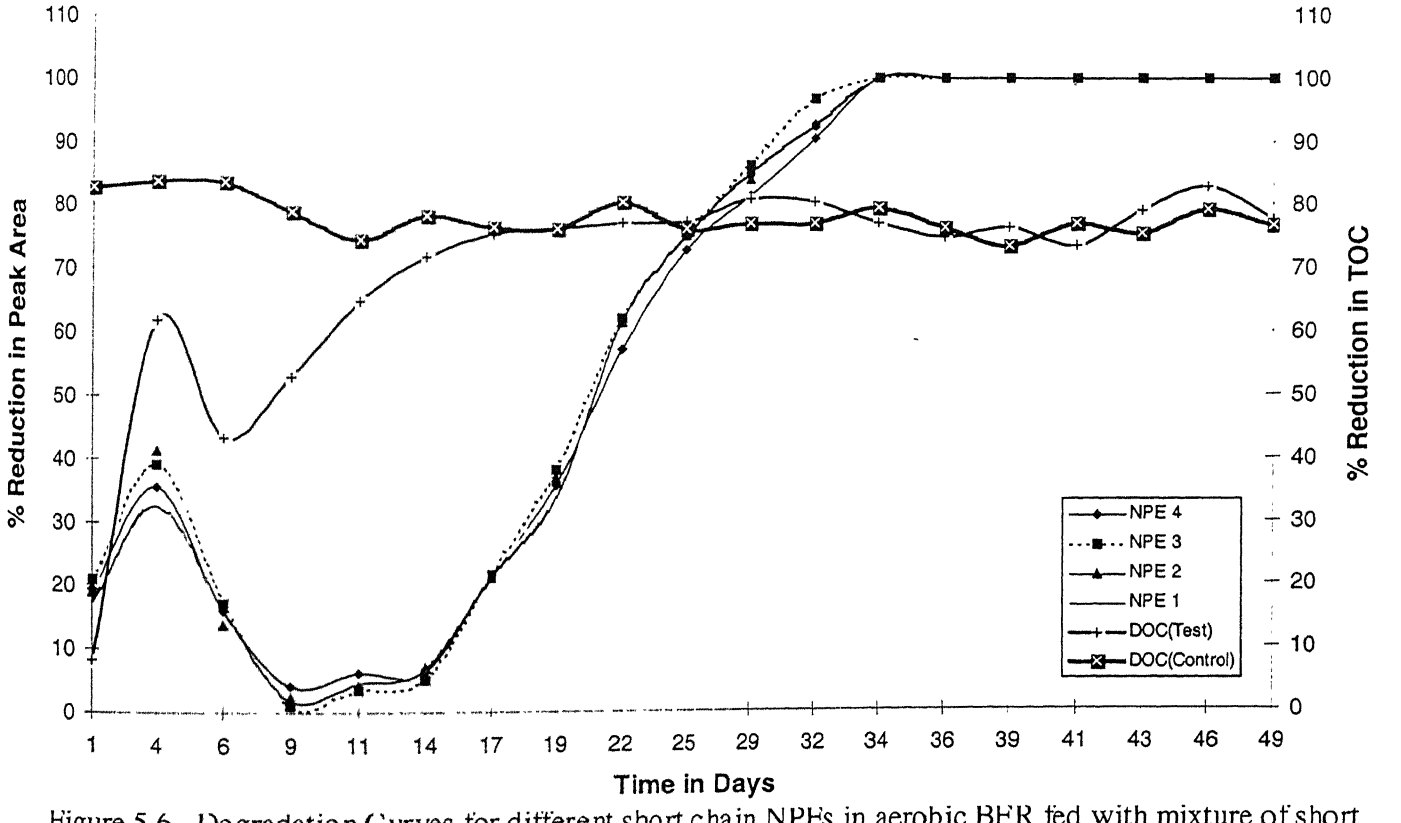


Figure 5.6. Degradation Curves for different short chain NPEs in aerobic BFR fed with mixture of short chain NPEs

- In the cases of OPEs and long chain NPEs, it was observed that no degradation of the target compounds took place up to 9th day and the increase in the overall DOC removal was slow until the 9th day and much more rapid thereafter. It is possible that for the short chain NPEs also, the actual biodegradation curve followed similar pattern. Since, short chain NPEs are more hydrophobic than the longer chain ones or the OPEs in Triton X 100, they rapidly accumulated on the surface of the biomass. It has been reported that these surfactants form hemi-micelles around the cells (Guha and Jaffee, 1996b). Because, of this initial accumulation in the reactors, there was an apparent removal of short chain NPEs. However, as with the breakthrough of surfactant accumulation in the reactor, the removal declined until the biodegradation took over starting from about day 9th. The accumulation of surfactants around the cell membrane will normally inhibit the release of exo-enzymes needed for degrading some of the complex compounds in the basal media. As a result, around the time of breakthrough of the surfactant, the DOC removal also attains a minimum. Once again, with the acclimation of cells and the induction of the enzymes capable of degrading the NPE compounds, both DOC and NPE degradation start to increase. There are also possibility of certain amount of damage to the cell walls due to formation of hemi-micelles and micelles that can sollubilize lipids.

A significant difference between the degradation performance of aerobic and anaerobic BFR was observed. The performance of anaerobic BFR was found to be better than that of aerobic BFR. In the last two weeks of the reactor operation, the aerobic reactor was seen to effect 75 – 80% (Figure 5.6) of the analyte removal, whereas, no analyte could be

detected in the anaerobic reactor effluents within the detection limits of the test system (Figure 5.7). This may be due to difference in cell surface hydrophobicity in the aerobic and the anaerobic cells. The anaerobic cells are reported to be more hydrophobic than the aerobic cells. The formation of hemi-micelles will be more on the more hydrophobic cells. The initial peak of removal, which is presumably due to surfactant adsorption (formation of hemi-micelles or ad-micelles), is higher in the case of anaerobic reactor (Figure 5.7) compared to that in the aerobic reactor (Figure 5.6). Once the cells are well adapted in utilizing the short chain NPEs, formation of hemi-micelles brings the substrates closer to the cells. This may explain near complete degradation of short chain NPEs in the anaerobic reactors.

5.2.4. Discussion

In all the cases studied above, both the DOC removal curves and the surfactant degradation curves for individual ethoxylates obtained from HPTLC analysis, shows an initial lag phase, irrespective of the analyte being tested for biodegradation by them. This was after the reactors were stabilized with the base feed which contributed 85% of the total DOC in the presence of APEs. The time to reach the second steady state was about 14 days, for DOC removal was about 30 days for the APEs. This may indicate that the initial inhibitory effect disappeared about two weeks but full enzyme induction takes about 30 days or the new population composition takes about 30 days to establish.

The microbes were pre-adapted to the degradation of base media and as a result, depletion of DOC takes speed earlier than the biodegradation of surfactants. This also shows that the biota is in the process of adapting to the new atmosphere and in the

meantime, depletion in the DOC of test solution, serves as the prime source of sustenance energy for the microbes in the biofilms. Microorganisms on developing the capacity to degrade the surfactants finally start degrading it and then continually increasing reduction in amount of surfactants is witnessed until a finally stable value of depletion in terms of percentage of influent is seen. At this stage the microorganisms in the biofilms were presumably in the stationary phase.

On comparing the DOC and surfactant degradation after the microbiota reaches the level of stability in degradation, it is observed that in all the cases the degradation of surfactant is more than the DOC. This can be justified as APEs are readily biodegradable provided their subjection to a well adapted microbiota, however, DOC is comprised of both degradable as well as recalcitrant compounds..

The biodegradation of APEs did not vary much with slight variation in the alkyl chain of the hydrophobic moiety (OPEs vs. long chain NPEs) but it was significantly different for the surfactants having the same hydrophobic moiety but different hydrophilic ethoxylate chain (long chain vs. short chain NPEs). It appears that in the compounds studied here, the number of ethylene oxide units in the hydrophilic chain guides the nature of biodegradation of the surfactant.

5.3. Degradation Products

The removal of test compounds in test BFRs for all the three mixtures was observed fairly soon but the appearance of degradation products was only witnessed after comaparatively longer time.

5.3.1. Degradation Products of Long Chain NonylPhenol Ethoxylates

The Figures 5.8 a, b and c shows the typical chromatograms of influent, effluent from aerobic reactor and effluent from anaerobic reactor, respectively. It may be observed that the peaks corresponding to the shorter chain NPEs (NPE 1 and NPE 2) are higher in the effluents than those in the influent. The Figures 5.9 and 5.10 shows the appearance of degradation products in the aerobic and anaerobic reactors, respectively. For quantification of the emergent compounds, the ratio of their peak area with the peak area of NPE 6 in Marlophen NP10 standard (applied on the same TLC plate), was used.

The appearance of degradation products was witnessed almost together in both aerobic and anaerobic BFRs. The degradation products were first observed on day 22^{nd} and increased with the days of operation until the end of the test period (50^{th} day). The short chain ethoxylates, mostly NPE 2 and NPE 1 were observed to be the major degradation products. This very well accorded with the suggested degradation pathway [Thiele, 1997] by shortening of the ethoxylate chains.

However, the presence of Nonylphenol (NP), devoid of any ethylene oxide unit, was not found in the effluents. It might be so that the compound was formed but in such small

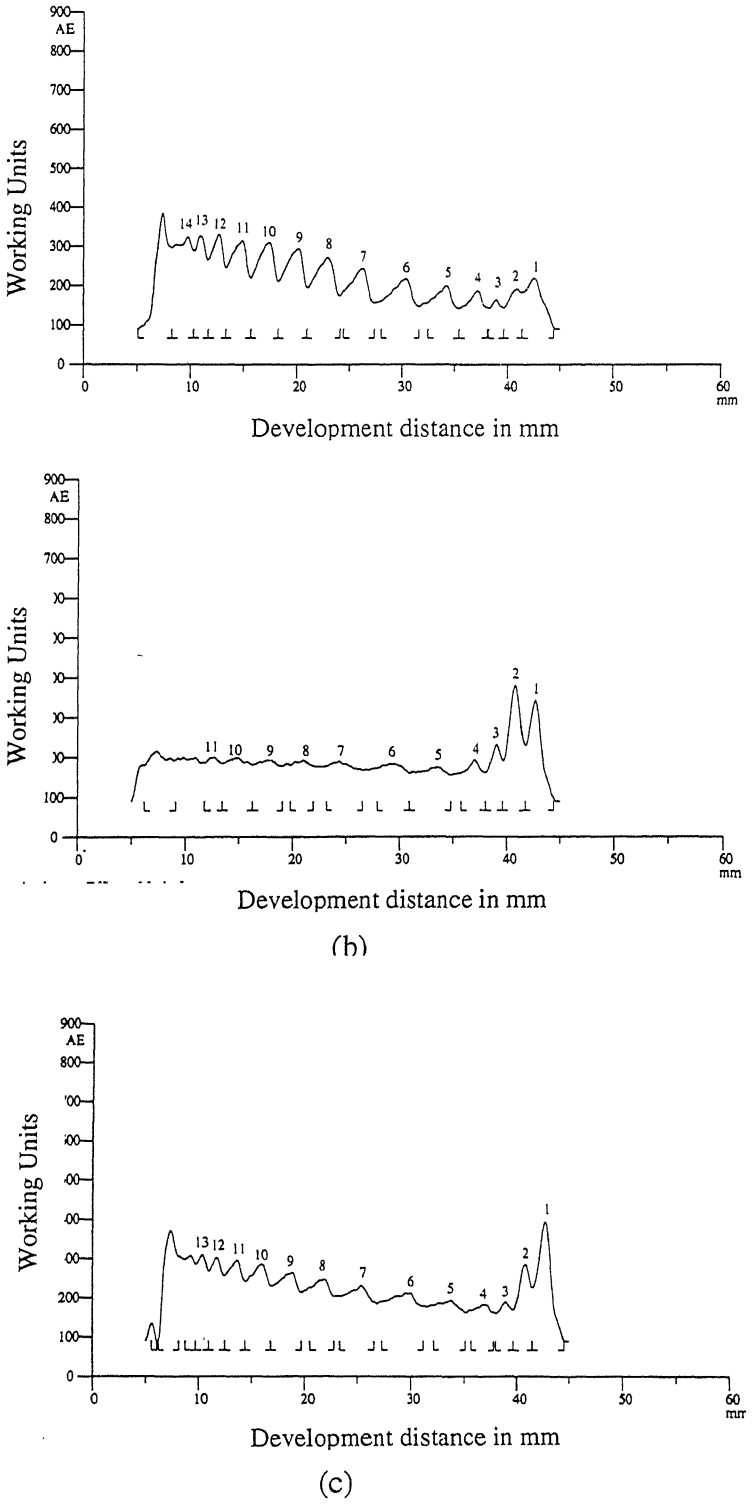


Figure 5.8. Chromatograms showing degradation of Long chain NPEs. (a) Influent to both aerobic and anaerobic BFR. (b) Effluent from aerobic BFR. (c) Effluent from anaerobic BFR

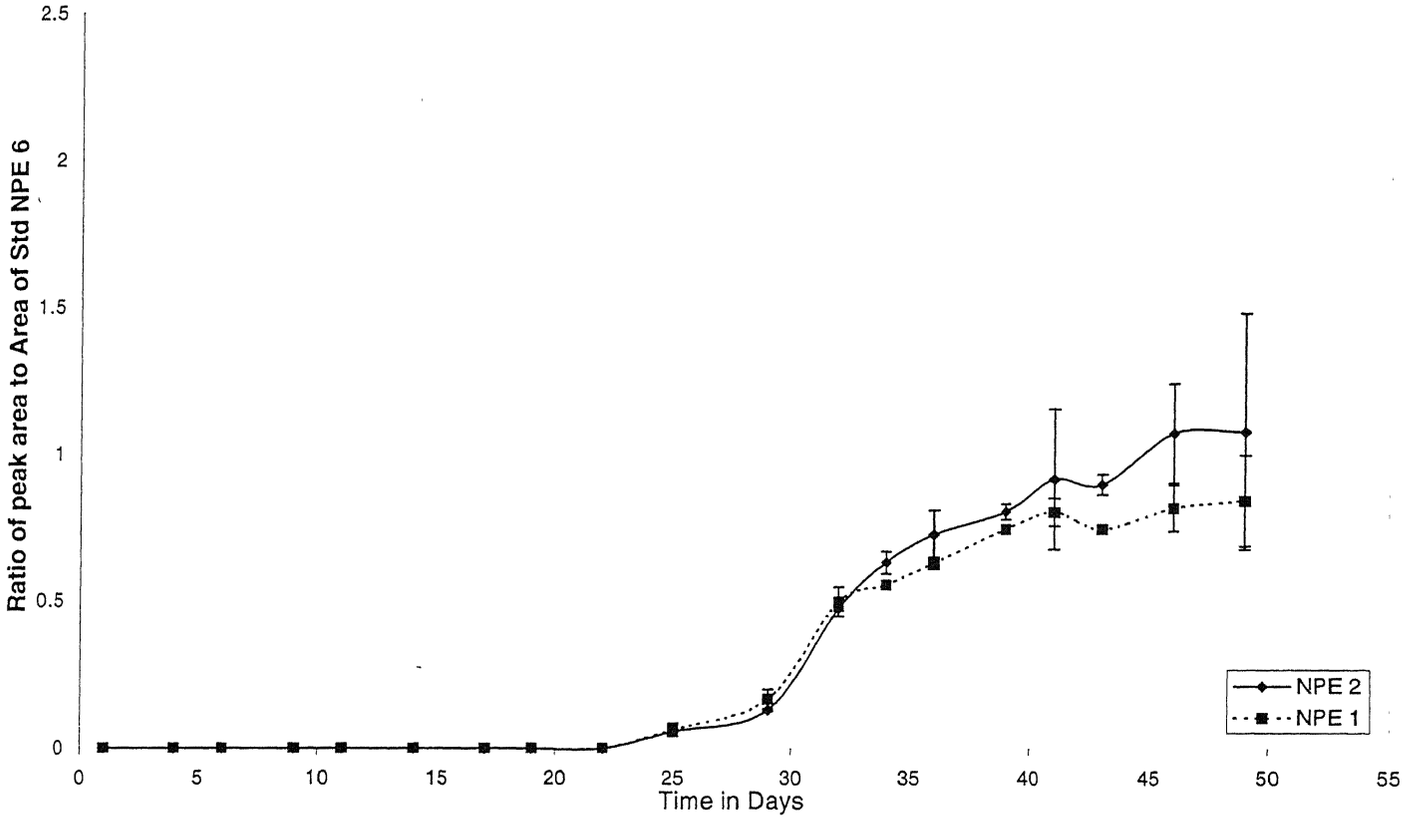


Figure 5.9. Emergence curves for compounds in aerobic BFR fed with mixture of long chain NPEs

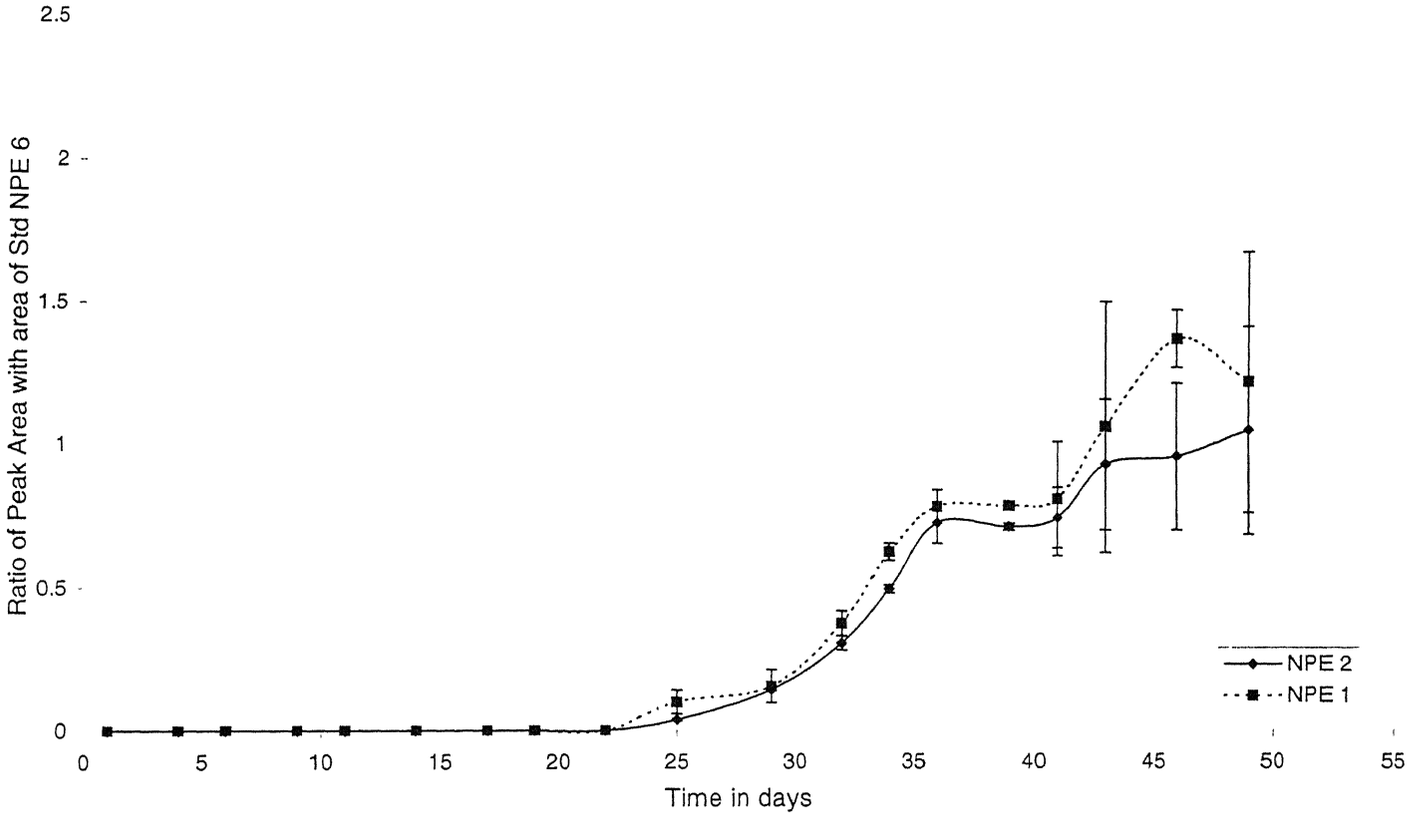


Figure 5.10. Emergence curves for compounds in anaerobic BFR fed with mixture of long chain NPEs

quantities so as to be non-detectable. Nonylphenol being highly hydrophobic may also be adsorbed to the cells.

The emergence curves for NPE 2 and NPE 1 with time in aerobic reactors suggested NPE 2 to be the dominant degradation product and the curves for anaerobic reactors suggested NPE 1 to be the dominant emerging product.

5.3.2. Degradation products of Octyl-Phenol Ethoxylates

The degradation products in case of Triton X-100 were also seen as rising peaks of shorter chain ethoxylates in the chromatograms accompanying the degradation of long chain ethoxylates. For quantitating the emergent compounds, the ratio of their peak area with the peak area of OPE 6 in Triton X-100 standard (applied on the same TLC plate), was used. Emergence of the degradation products with time is shown in the Figures 5.11 and 5.12 respectively, for the aerobic and anaerobic reactors. Unlike the case of long chain NPE degradation, the degradation products did not appear simultaneously in aerobic and anaerobic reactor. The appearance of degradation products was witnessed in aerobic reactor about 5 days earlier than the anaerobic reactor.

The shorter chain ethoxylates, mostly OPE 1 and OPE 2 were found as the major emergent compounds. The amount of OPE 1 produced in the anaerobic BFR (Figure 5.12) was much more than that produced in the aerobic BFR (Figure 5.11). In addition, OPE1 is clearly the main degradation product in the anaerobic BFRs. No signs of presence of Octylphenol (OP) in the effluents were observed from the chromatograms, again leading us to the conclude that either AP were not produced at all due to low

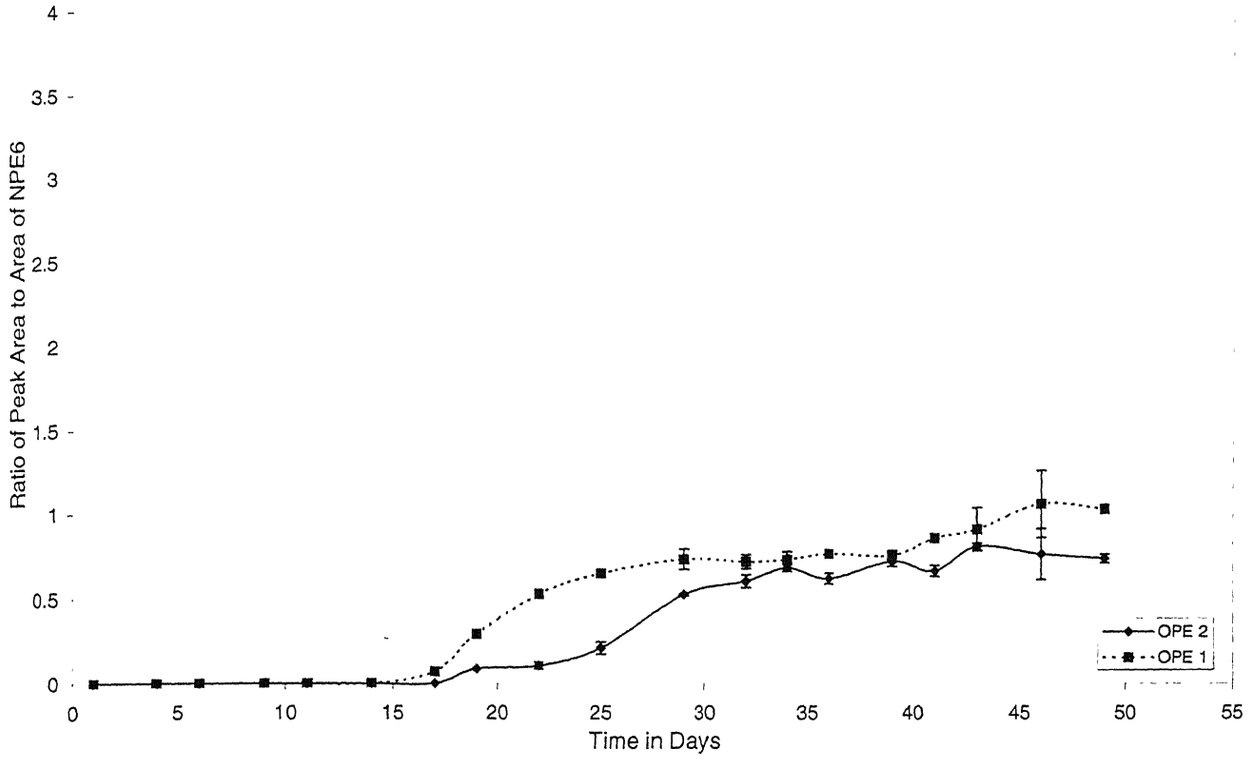


Figure 5.11. Emergence curves for compounds in aerobic BFR fed with mixture of long chain OPEs

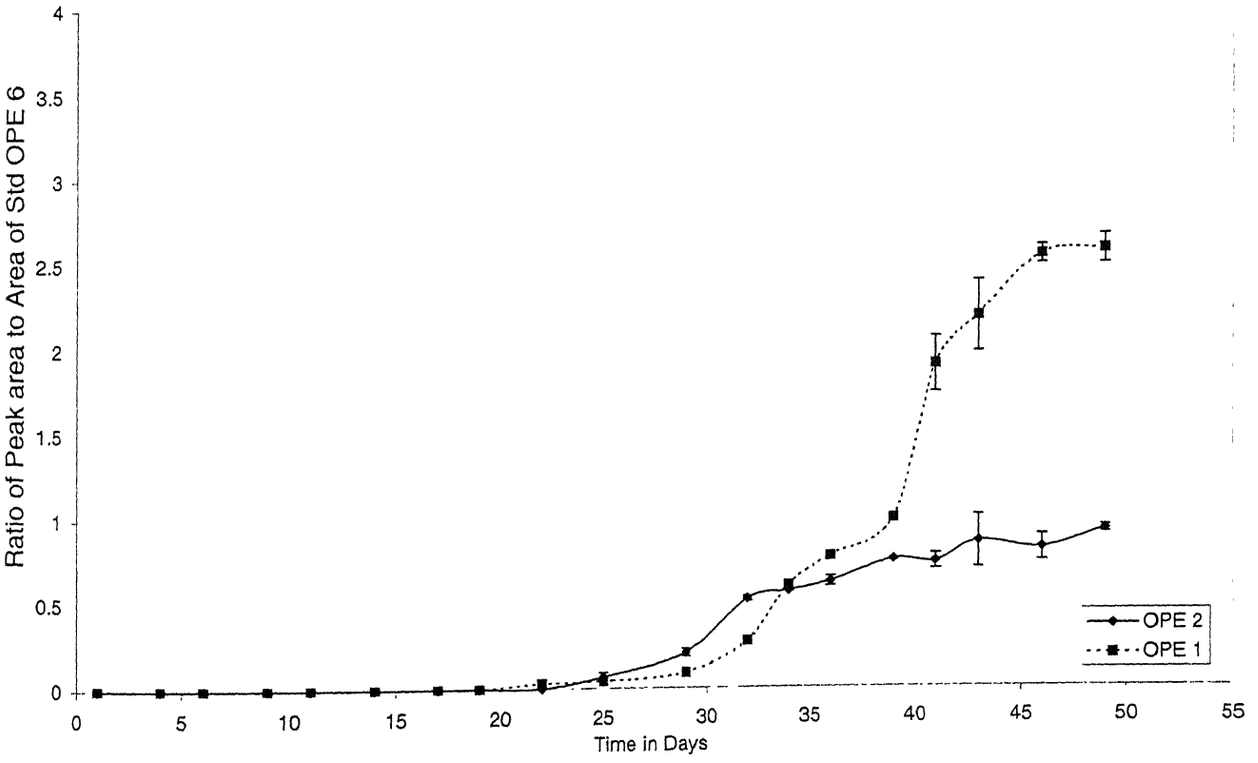


Figure 5.12. Emergence curves for compounds in anaerobic BFR fed with mixture of long chain OPEs

retention time of the test compounds in the BFRs or if produced they were in such small quantities or adsorbed onto the cell surface so as to be undetectable in the aqueous phase.

5.3.3. Degradation Products of Marlophen NP3

Although very large efficiency of degradation was achieved for test reactors fed with Marlophen NP3 after the initial acclimation period, no degradation product could be detected for the degradation of short chain ethoxylates. The compound removal efficiency was much higher in anaerobic reactors but new compound or emergent peaks could not be seen with the instrument and method being used.

5.3.4. Tests for Carboxylate Derivatives of the Analytes

Knowing the high probability of APEs getting converted to their respective carboxylate derivatives as suggested extensively in the literature, efforts were made to detect the carboxylate derivatives, if any, formed in the test reactors. In order to accomplish this objective the influent and effluent samples were acidified with HCl prior to SPE and the extracts were then analysed with HPTLC.

No Alkylphenol Ethoxycarboxylates (APEC) could be detected, within our detection limits for any of the three test compounds. This made us derive to the conclusion that either APECs were not produced in our system due to low retention time of the test compounds in the reactors or it was produced but in so less quantity that it was not possible to detect with the HPTLC system.

5.4. *Investigations on Cell Activity and the Types of Bacteria in Biofilms*

5.4.1. Determination of Types of Bacteria in Biofilms

The samples from the control and long chain NPE test reactors (both aerobic and anaerobic) were analysed using FISH technique to investigate the major types of bacteria dominating in the bacterial consortium in the biofilms. The majority of bacteria when compared to the whole cell number, found in both aerobic and anaerobic (control and test) BFRs were seen to be Eubacteria (Figure 5.13.a). No signs of Archeabacteria were seen in aerobic BFRs, however, loose binding with the probe for Archeabacteria were seen in the samples from Anaerobic BFRs (Figure 5.13.b), thereby, deriving to conclusion that no archeabacteria were present in aerobic BFRs, however, they might be present in anaerobic BFRs but in very low numbers.

Further, samples were tested for the largest phyllum of Eubacteria viz. Proteobacteria (441 Genera) which is further divided into 5 classes. Analysis was done using probes for 3 of its classes viz. Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria. Analysis for the remaining 2 classes could not be done due to non-availability of their respective probes.

The analysis results suggested dominance of Betaproteobacteria followed by Alphaproteobacteria and tailed by Gammaproteobacteria (Figure 5.14 b, c and d) in the initial phases of experimentation prior to feeding of the APEs to the test reactors. However, no differences in the bacteria types in aerobic and anaerobic BFRs was seen.

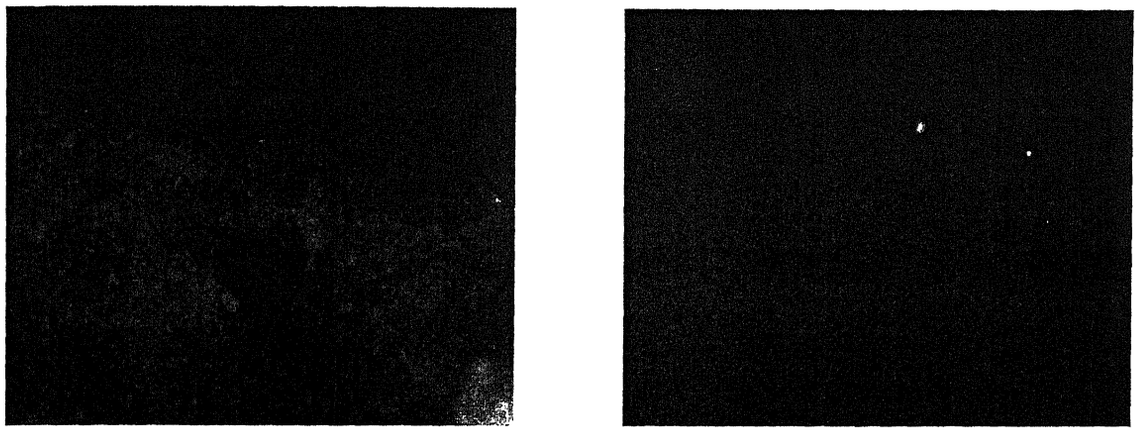


Figure 5.13. Eubacteria and Archeabacteria found in samples (a) Samples showing binding with probe for Eubacteria. (b) samples from anaerobic reactors showing binding with probe for Archeabacteria

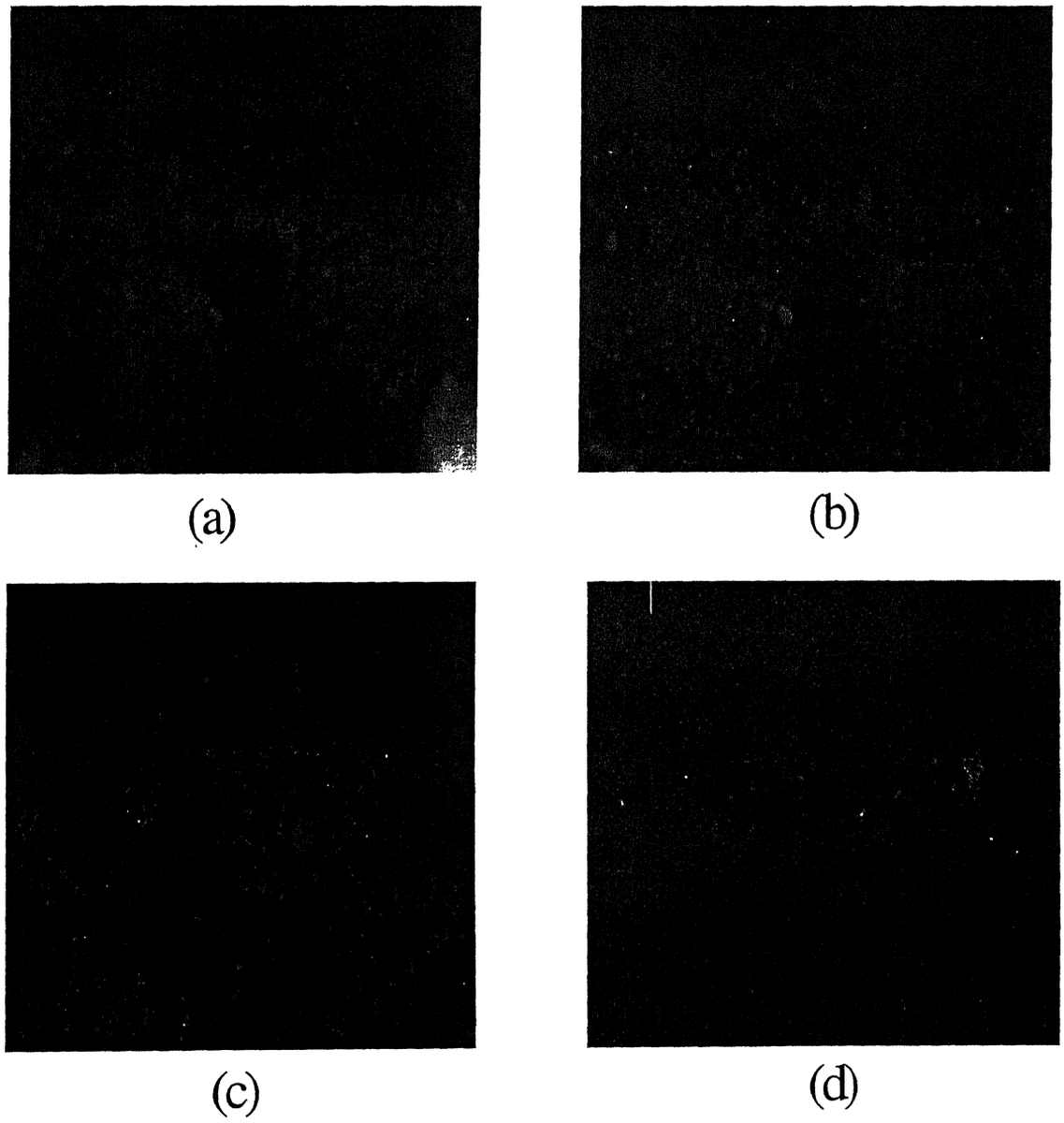


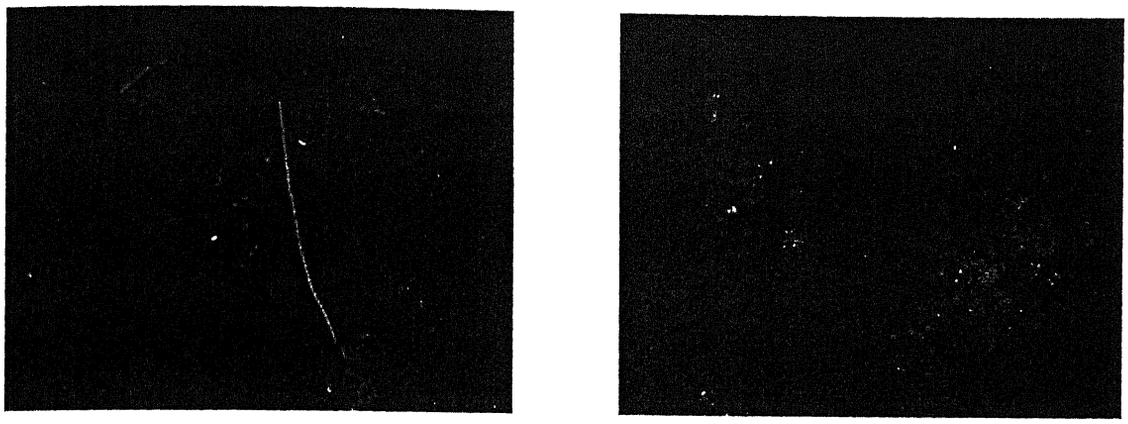
Figure 5.14. Types of cells in BFRs. (a) Eubacteria in biofilm samples. (b)Alphaproteobacteria in biofilm samples. (c) Betaproteobacteria in biofilm samples. (d) Gammaproteobacteria in biofilm samples

In the later phases after the test reactors were acclimatized to the APEs (as deduced from the DOC degradation and HPTLC tests) no major change was observed in the composition of the consortium. However, the investigations being done with probes for broad categories of bacteria, did not give any clue to changes in the dominant genera and species of bacteria in the consortium in biofilms.

All the three types of proteobacteria viz. Alpha, Beta and Gamma type Proteobacteria were again found to be present in the second phase of analysis. In the later phases, the Alphaproteobacteria were found to compete with the Betaproteobacteria both in aerobic and anaerobic (control and test) reactors. But the Gammaproteobacteria did not show changes in their population with respect to time in any of the reactors. This lead to conclusion that the Alphaproteobacteria required more time to get adapted to the conditions in the BFRs. However, the changes being observed in both control and test reactors could not be attributed to the introduction of APEs in the test system. The major difference observed between aerobic and anaerobic BFRs (both control and test) was the presence of long chains of bacteria in the aerobic reactors which could not be detected in the anaerobic reactors (Figure 5.15. a and b)

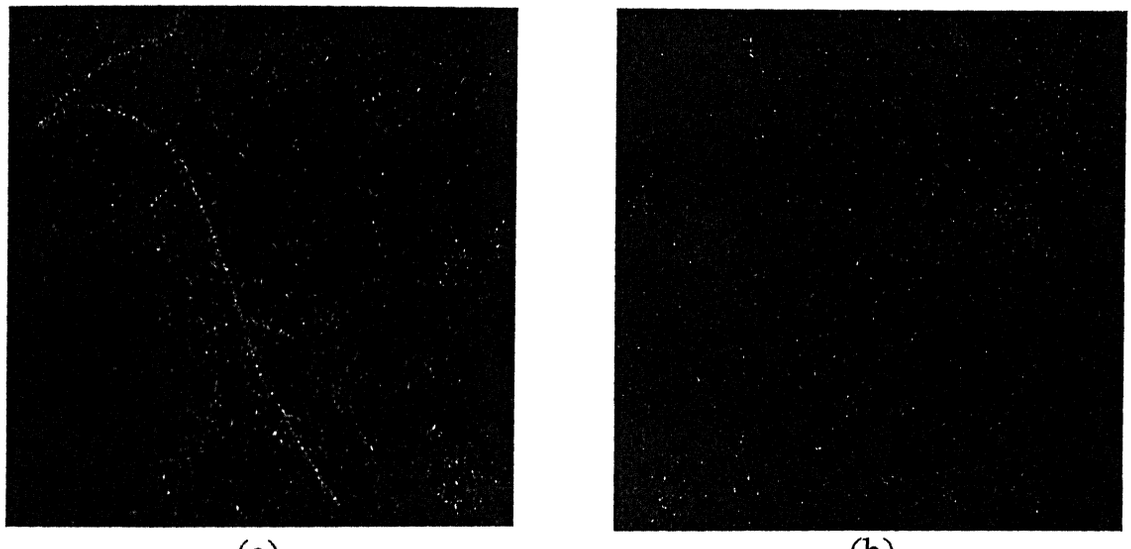
5.4.2. Determination of Cell Activity in BFRs

The CTC scanning for cell activity in the samples was also performed after fixing cells from the samples on Millipore membranes without fluorescence indicator (0.2 mm GTBP). The CTC itself being a water soluble, oxidized compound gets reduced in the active cells to form red colored water insoluble crystals thereby, demarcating the active cells on the membrane which are observed with the help of DAPI, a DNA binding dye.



(a) (b)

Figure 5.15. Eubacteria in aerobic and anaerobic reactors. (a) Eubacteria in aerobic reactors. (b) Eubacteria in Anaerobic reactors



(a) (b)

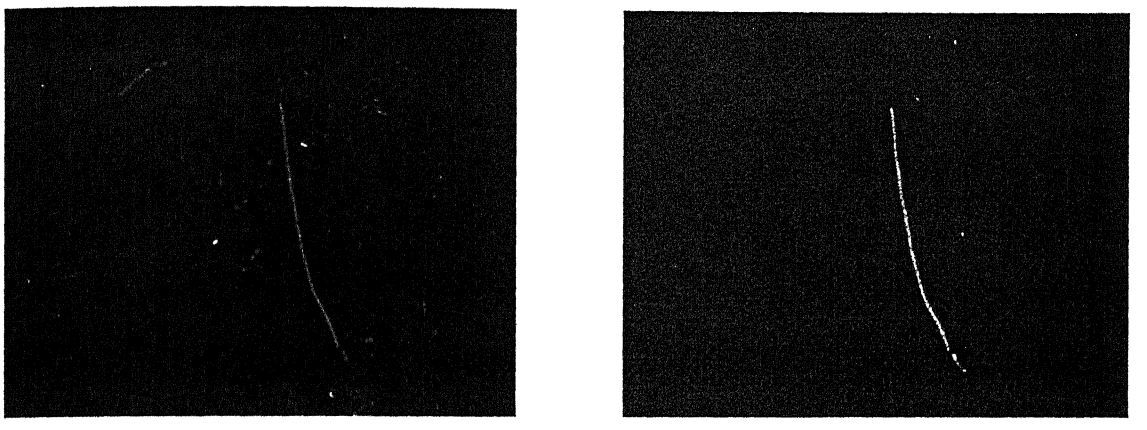
Figure 5.16. Active cells in aerobic and anaerobic BFRs. (a) Active cells concentrated in chains and flocs in aerobic BFRs. (b) Active cells dispersed as single cells in anaerobic BFRs

The results from CTC scanning, suggested both the aerobic and anaerobic reactors to be composed of high density of active cells. After the acclimation to analyte, the test reactors were observed to be slightly more active than the control reactors. However, in the second phase of analysis also, no changes in cell activity, among aerobic and anaerobic BFRs (both control and test) was observed.

In the aerobic reactors, the active cells were found to be concentrated in the chains and flocks and not many single cells were seen to be active. On the contradictory, in the anaerobic reactors, no such chains and flocks composed of active cells could be observed (Figure 5.16. a and b)

5.4.3. Chains in Aerobic Biofilm Reactors

The chains were exclusively found to be present in the aerobic reactors. Two different types of chains were observed – one having big round cocci and the second type comprising of small bacilli (rod shaped cells). The analysis results revealed that both the chain types showed binding with the probe for Eubacteria. However, on investigating further with three types of probes for Alpha, beta and Gamma type Proteobacteria, only chains with rod shaped cells showed binding with probe for Gammaproteobacteria, whereas, the chains with round cells did not bind to any of the available Proteobacteria probes (Figure 5.17, a and b) This lead to conclusions, that aerobic reactors, have two types of chains of Eubacteria – one type of chains being Gammaproteobacteria while the other type albeit Eubacteria, but not any of the Gram negative Proteobacteria types. However, no differences between types of chains were observed between control and test aerobic BFRs.



(a) (b)

Figure 5.17, Two types of chains in Aerobic BFRs. (a) Two different types of chains binding with probe for Eubacteria. (b) Only one type of chain showing binding with probe for Gammaproteobacteria

5.5. Adsorption of the APEs on the Biofilms in BFRs

5.5.1. Weight of the biomass in BFRs

The weight of biomass present in the aerobic BFRs of all the three test compounds were generally found to be higher than the weight of biomass present in the respective anaerobic reactors as can be seen from Figure 5.18.

Larger weight of biomass in aerobic BFRs is in well accordance with the fact that the ratio of maintenance energy to reproduction energy is much higher for anaerobic microorganisms as compared to aerobic microorganisms, thereby, leading to less amount of biomass formation for anaerobes as compared to aerobes when fed with the same amount of nutrients. However, presence of substantial amount of biomass in all the reactors but for Marlophen NP10, anaerobic test reactor, leads to the conclusion that in the given concentration the APEs were not toxic to the bacteria in the biofilms. The abnormally low mass in Marlophen NP10 anaerobic test reactor might be attributed to the experimental errors or to the accumulation of toxic metabolites in the reactor, which killed a part of the biofilm.

5.5.2. Adsorbed APEs on Biofilms

The extraction of biofilms in the reactors was done with the polar solvent (Methanol) to extract the long chain ethoxylates and with a non-polar solvent to extract the non-polar, short chain APEs.

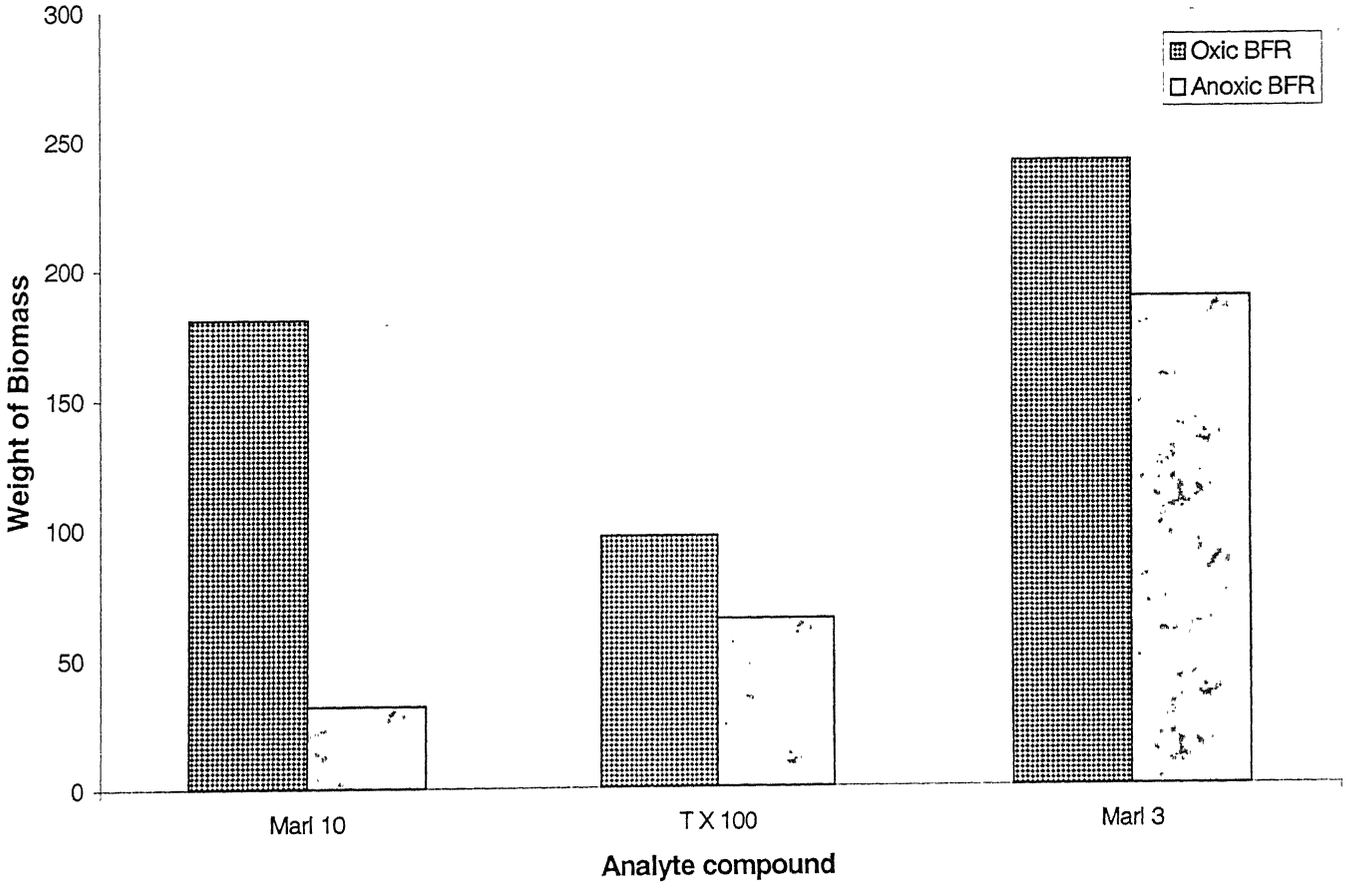


Figure 5.18. Weight of biomass in BFRs for degradation test of three analytes

It was not possible to detect any of the long chain ethoxylates in any of the biofilm extracts due to presence of large amount of interference. The interfering peaks might be a result of microbial debris that also got extracted in the solvents.

However, in general, for all the biofilm reactors APE1 and AP were observed adsorbed on the biofilm surface. Not much difference was observed in the adsorbed metabolites among aerobic and anaerobic reactors for one analyte. The majority of APE1 and AP were extracted in Hexane but for Marlophen NP10 test reactors where a little amount of both the NPE1 and NP were found to be present in methanol as well.

It was not possible to quantify the amount of adsorbed OPE 1 due to unavailability of OPE1 Standard. Apart from that, the rest of the adsorbed metabolites as quantified by comparing with standards calibration curve made using OPE 1 standards of 0.1, 0.5, 1.0, 2.0 and 3.0 μg can be seen in Table 5.2, Table 5.3 and Table 5.4.

It is notable that the alkylphenols were not detected in the effluents from the BFRs. However, they were found adsorbed to the surface of biofilm. This might be because alkylphenols were produced in extremely small amounts as degradation product of APE degradation. Moreover, due to their highly lipophilic character, they were retained onto the surface of the biomass in the BFRs where they accumulated in considerable amounts, easily detectable with the HPTLC system.

Test Reactor	NPE1			NP		
	Hexane	Methanol	Total	Hexane	Methanol	Total
Aerobic	773.47	123.38	896.85	602.32	121.58	723.90
Anaerobic	268.21	35.15	303.36	202.11	42.14	244.25

Table 5.2. Weight of adsorbed metabolites in Marlophen NP10 test reactors.(in ng)

Test Reactor	NPE 1			NP		
	Hexane	Methanol	Total	Hexane	Methanol	Total
Aerobic	478.61	-	478.61	542.32	-	542.32
Anaerobic	410.22	-	410.22	390.26	-	390.26

Table 5.3. Weight of adsorbed metabolites in Marlophen NP3 test reactors.(in ng)

Test Reactor	OP		
	Hexane	Methanol	Total
Aerobic	320.65	-	320.65
Anaerobic	448.19	-	448.19

Table 5.4. Weight of adsorbed metabolites in Triton X-100 test reactors.(in ng).

It is also notable that the amounts of NPE1 and NP adsorbed on the biofilms of Marlophen NP10 test reactors were more than that adsorbed on biofilms of Marlophen NP3 test biofilm reactors. This result leads to conclusion that the microbes in Marlophen NP10 test reactors were acclimated fairly well to the long chain ethoxylates but were not so efficient in degrading shorter NPEs. However, the microbes in the Marlophen NP3 fed reactors being fed with shorter ethoxylates had well adopted to the shorter NPEs and hence their higher degradation efficiency justifying the presence of low quantity of metabolites on their surfaces.

Chapter 6

Conclusions

Good biodegradation of long chain ethoxylates was witnessed in both aerobic and anaerobic BFRs following 2 weeks of acclimation period. The short chain ethoxylates were detected as the major degradation products of long chain APEs, both for OPEs and NPEs. This suggested the shortening of ethoxylate chain as the dominant biodegradation pathway for APE degradation. Mostly APE 2 and APE 1 were found to be the dominant emergent compounds.

The BFRs subjected to exposure with short chain NPEs were also found to degrade the shorter ethoxylates after a two weeks acclimation period. However, no degradation product could be seen for the shorter ethoxylates. Interestingly, the anaerobic reactor was found to be more efficient in degradation of short chain ethoxylates than the aerobic reactors.

No signs of alkylphenols (without any ethoxylate unit) could be observed in both aerobic and anaerobic reactor effluents for any of the test compounds. However, small amount of alkylphenols were recovered from the biomass after the reactors were dismantled. This leads to conclusion that the alkylphenols were produced in small quantities and, being highly hydrophobic, were adsorbed onto the biomass.

No carboxylate derivatives of APEs were detected in the BFR effluents presumably due to the low retention time of APEs in the BFRs was not conducive to their formation.

The DOC degradation studies also revealed the same trends of DOC degradation as that of individual ethoxylate degradation. An overall 80 – 85% DOC removal was witnessed for long chain NPEs and OPEs. DOC removal of about 75 - 80% was seen in aerobic BFR for short chain NPEs and about 85 –90% for anaerobic BFRs. However, it was noticed after

reactor acclimatization that the % removal of DOC (contributed together by base medium and APEs) was less than the % removal of individual ethoxylate compounds indicating that the APEs were the preferred substrate.

The FISH results revealed Eubacteria to be the dominant category of bacteria in the BFRs. A small presence of Archeabacteria was observed in the anaerobic BFRs, but none were seen in the aerobic BFRs. In the initial phases, maximum number of Betaproteobacteria followed by Alphaproteobacteria and tailed by Gammaproteobacteria was observed. In later phases almost the same number of Alpha and Beta type Proteobacteria were observed both in test and control BFRs leading to conclusion that Alphaproteobacteria required some time to adapt to the reactor conditions. However, no changes in the cell types of the bacterial community were observed after feeding the test reactors with the analyte compound.

Both aerobic and anaerobic BFRs exhibited good cell activity. No changes in the cell activity were observed before and after feeding the APEs to the reactor. This indicates that the APEs were not toxic to the existing biofilms.

In a nutshell, it can be stated that short chain ethoxylates were more recalcitrant than longer chain APEs, however, if subjected to well acclimatized micro biota, they can also be fairly well biodegraded. The production of non-polar, lipophilic short chain ethoxylates as metabolites of degradation of longer APEs still puts a question mark to the environmental acceptability of APEs as surfactants.

Chapter 7

Scope forFuture Work

The following areas are recommended for future investigations –

- The effects of biodegradation on estrogenic potential of the APEs may be taken up for investigation.

- Investigations may be done to know the predominant site of biodegradation of APEs on the bacterial cell. This would also add up to the knowledge about the information about the kind of bacterial enzymes, *i.e.*, the extra cellular or the intracellular enzymes, involved in biodegradation.

- A more detailed analyses of the microbial consortia forming the biomass adapted for APE degradation, if pursued, would also help to enlighten more facts about APE biodegradation and would render us capable to select the microbes for bioengeening them to suit the ideal APE degradation pathway.

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Appendix

1. *List of the chamicals used in APE biodegradation analyses*

Chemical	***Manufacturer***	***%Purity***
Triton X-100	Sigma	'Technical product'
Marlophene NP10	Condea	'Technical Product'
Marlophene NP3	Condea	'Technical Product'
Methanol	Merck	Min.99.8%
Hexane	Merck	Min 99%
Dichloromethane	J.T.Baker	>99%
NaCl	Merck	Min99.5%
Peptone from Caesin	Merck	
Meat Extract	Merck	
Urea ($NH_2.CO.NH_2$)	Merck	Min 99.5%
$CaCl_2.2H_2O$	Merck	Min 99.5%
$MgSO_4.7H_2O$	Merck	Min 99.5%
K_2HPO_4	Merck	98 – 101%
Mucasol (Cleaning Solution)	Merz	
Acetone	J.T.Baker	>99.7%
Parafin	Merck	
Ethyl Acetate	Merck	

2. *List of Instruments used in biodegradation analyses of surfactants.*

Instrument	Manufacturer	Model	Other Details
Silica Gel KG60,HPTLC plates	Merck		10x20cm, without Fluorescence indicator
Automatic TLC Sampler	Camag	ATS TLC Sampler III	
Automated Multiple Development Instrument	Camag	Camag AMD 2	
Chromatogram Immersion Device	Camag	Camag Chromatogram Immersion device III	
Thil Layer Chromatography Scanner	Camag	Camag TLC Scanner 3	With Multiple wavelength detector and Cut off filters.
Shimadzu TOC Analyser	Shimadzu Corp.	TOC –5000A	With Autosampler,ASI-5000A
TCS Trocken – temperiersystem	Labor Technik Barkey		N_2 drying system
Water Pump	Ismatec		
Pump Tubings	Tygon		1.85 and 0.95 mm i.d.
SPE cartridges	Oasis		HLB, 6cc (200mg)
pH Meter	WTW	pH 325	
Magnetic Stirrer	Heidolph		
Weighing Machine	Sartorius		0.00001g accuracy
0.45µm Membrane Filter (Cellulose nitrate, d = 100mm)	Sartorius		Filteration of Sludge from Wasewater treatment plant
Isopore membrane Filters	Millipore		0.4µm and 0.2µm pore size
Pipettes	Hirschmann		5, 10, 20,25, 50mL
Micropipettes	Brand		10 –100 µL 100 – 1000 µL 0.5 – 5 mL
Flasks	IDL, Brand		5, 10, 20, 50, 100, 250, 500 and 1000 mL
Beakers	Schott		25, 50,100, 200, 500 mL

3. *Solvent composition and development distance for the steps involved in chromatogram development by AMD.*

Step No.	Development Distance	Ethyl Acetate + Water	Acetone	n-Hexane
1	10	100	0	0
2	12	95	5	0
3	14	90	10	0
4	16	85	15	0
5	18	80	20	0
6	20	70	30	0
7	22	60	40	0
8	24	50	50	0
9	26	40	60	0
10	28	30	70	0
11	30	20	80	0
12	32	0	100	0
13	34	0	100	0
14	36	0	90	10
15	38	0	80	20
16	40	0	70	30
17	42	0	60	40
18	44	0	50	50
19	46	0	40	60
20	48	0	30	70
21	50	0	20	80
22	52	0	0	100